

**A Layer of Hydrogel on PET Dressings for Low Wound  
Adherence and as a Reservoir for New Dendrimer Based Biocides  
for Burn Wound Infection Control**

By

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## ABSTRACT

Bacterial infection in burn wounds can jeopardize healing and can even lead to a patient's death. Recently using a topical wound dressing with antimicrobial ability has been increasing. An ideal dressing for a burn wound is required to be antimicrobial, pain free and able to control the wound moisture to facilitate healing. In this research, a UV-radiation grafting method for depositing polyacrylamide (PAM) layer was used to improve the non-adherence properties of two commercially available silver based wound dressings. The dressing adherence, evaluated via an *in vitro* gelatine model, dramatically decreases after the deposition of PAM. This deposition did not negatively affect the antibacterial ability and cytotoxicity of the dressings. Furthermore, a poly(amidoamine) dendrimer based biocide was incorporated into PET dressings coated with poly(acrylic acid-co-acrylamide ) hydrogel. The resulting dressing was able to release a therapeutic dose of the antimicrobial agent in a sustained manner.

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# TABLE OF CONTENTS

ABSTRACT.....	I
ACKNOWLEDGEMENTS.....	II
TABLE OF CONTENTS .....	III
LIST OF TABLES.....	V
LIST OF FIGURES.....	VI
LIST OF SCHEMES.....	IX
LIST OF ABBREVIATIONS: .....	XI
CHAPTER 1. INTRODUCTION.....	2
1.1 OVERVIEW.....	2
1.2 BURN INJURY .....	3
1.3 WOUND CARE AND USAGE OF WOUND DRESSING FOR BURN WOUNDS.....	4
1.3.1 Absorption and moisture retain dressings.....	6
1.3.2 Antimicrobial wound dressings.....	6
1.3.2.1 Silver .....	7
1.3.2.2 Iodine.....	8
1.3.2.3 Polybiguanides .....	9
1.3.2.4 Honey .....	10
1.3.2.5 Advantages and disadvantages .....	10
1.3.2.6 Evaluation of antimicrobial efficacy .....	12
1.3.3 Wound adherence of burn wound dressings.....	14
1.3.3.1 Advantages and disadvantages of non-adherent and atraumatic dressings.....	15
1.3.3.2 Evaluation of adhesive dressings .....	16
1.4 HYDROGEL AS A MORE PROMISING MATERIAL FOR BURN WOUND DRESSINGS .....	18
1.4.1 Hydrogel preparation.....	19
1.4.2 Hydrogel in drug delivery.....	20
1.4.2.1 Tuning hydrogel to achieve controlled release.....	21
CHAPTER 2. HYPOTHESIS AND OBJECTIVES .....	26
CHAPTER 3. MATERIALS AND EXPERIMENTS .....	29
3.1 MATERIALS.....	29
3.2 EXPERIMENTAL PART 1.....	29
3.2.1 Sample preparation and polyacrylamide (PAM) hydrogel deposition.....	29
3.2.2 Swelling ratio:.....	30
3.2.3 Peeling force test.....	31
3.2.4 Disk Diffusion test .....	31
3.2.5 Suspension test .....	32

3.2.6	<i>Silver release test</i> .....	32
3.2.7	<i>Cytotoxicity test</i> .....	33
3.3	EXPERIMENTAL PART 2.....	34
3.3.1	<i>Sample preparation</i> .....	34
3.3.2	<i>Weight increment and swelling ratio</i> .....	35
3.3.3	<i>Determination of carboxylic acid content on samples</i> .....	35
3.3.4	<i>Peeling force test</i> .....	36
3.3.5	<i>Synthesizing a new biocides</i> .....	37
3.3.6	<i>Loading compound QASG1C12 on PET hydrogels</i> .....	37
3.3.7	<i>Release studies</i> .....	38
3.3.7.1	Spectrophotometric method of drug release measurement.....	38
3.3.7.2	Release behavior analysis.....	38
3.3.8	<i>Disk Diffusion test</i> .....	39
3.4	STATISTICAL ANALYSIS.....	40
3.5	SIGNIFICANCE:.....	40
<b>CHAPTER 4. RESULTS AND DISCUSSIONS</b> .....		<b>42</b>
4.1	PART 1.....	42
4.1.1	<i>Peeling force test</i> .....	42
4.1.2	<i>Antimicrobial assays</i> .....	45
4.1.3	<i>Silver release test</i> .....	50
4.1.4	<i>Cytotoxicity test</i> .....	52
4.1.5	<i>Effect of autoclaving on peeling energy and antibacterial ability of samples</i> .....	55
4.1.6	<i>Effect of ethylene oxide sterilization on peeling energy antibacterial ability of samples</i> .....	59
4.2	PART 2.....	62
4.2.1	<i>Deposition of acrylamide-co-acrylic acid hydrogel on PET</i> .....	62
4.2.1.1	Determination of carboxylic acid content on samples.....	64
4.2.1.2	Peeling energy test.....	66
4.2.2	<i>Preparing antimicrobial dressing- Loading and release of biocide</i> .....	68
4.2.2.1	Disk diffusion assay.....	73
<b>CHAPTER 5. SUMMARY AND CONCLUSION</b> .....		<b>78</b>
<b>BIBLIOGRAPHY</b> .....		<b>83</b>

## LIST OF TABLES

<i>Table 1.</i>	Acrylamide/acrylic acid monomer preparation .....	35
<i>Table 2.</i>	Zone of inhibition diameters (mm) of dressings tested against MDR P.aeruginosa and CA-MRSA. The resulting concentrations were respectively 1.00E+08 and 1.29E+08 (CFU/mL) for MDR P.aeruginosa and CA- MRSA. ....	47
<i>Table 3.</i>	Zone of inhibition diameters (mm) of dressings tested against MDR P.aeruginosa and CA- MRSA with the edge coated samples .....	48
<i>Table 4.</i>	Antimicrobial efficiency of dressings tested against CA-MRSA.....	49
<i>Table 5.</i>	Antimicrobial efficiency of dressings tested against MDR P.aeruginosa.....	50
<i>Table 6.</i>	Weight increment of samples and normalized swelling ratio (%).....	64
<i>Table 7.</i>	Release kinetics from samples.....	71
<i>Table 8.</i>	ZOI diameter (mm) of dressings against MDR P.aeruginosa and CA-MRSA ....	74

## LIST OF FIGURES

- Figure 1.* Comparing effect of monomer concentrations on swelling ratio and peeling energy of PET treated samples. Double asterisks indicate swelling ratio and peeling energy values for acrylamide treated samples are significantly different from those of PET-PAM-10. ( $p < 0.05$ )..... 43
- Figure 2.* Comparing effect of pressure on swelling ratio and peeling energy of Acticoat treated samples. Double asterisks indicate swelling ratio and peeling energy values for acrylamide treated samples are significantly different from those of Acticoat-PAM-10. ( $p < 0.05$ ) ..... 43
- Figure 3.* Comparing peeling energy of treated and untreated dressings. Double asterisks significant difference between peeling energy of PAM-10 treated sample and its untreated counterpart ( $p < 0.05$ ). ..... 44
- Figure 4.* Comparing cumulative silver release concentration of Acticoat Flex 3 samples after 30 minutes, 2 hours and 48 hours. Double asterisks indicates significant difference between PAM treated and untreated values at each time interval ( $p < 0.05$ ,  $n = 3$ ). ..... 51
- Figure 5.* Evaluation of relative Fibroblast cell viability after 24 hours dressing treatment. The exhibited results are percentage of viable cells of untreated Acticoat and Silverlon dressings or polyacrylamide grafted dressings to the control group both in the culturing media containing saline or water. Double asterisks indicates significant difference between PAM treated and untreated values in each culturing media ( $p < 0.05$ ,  $n = 12$ ). ..... 53
- Figure 6.* Evaluation of relative keratinocyte cell viability after 24 hours dressing treatment. The exhibited results are percentage of viable cells of untreated Acticoat and Silverlon dressings or polyacrylamide grafted dressings to the control group both in the culturing media containing saline or water. Double asterisks indicates significant difference between PAM treated and untreated values in each culturing media ( $p < 0.05$ ,  $n = 12$ ). ..... 54
- Figure 7.* Comparing peeling energy of autoclaved samples with not-autoclaved samples 56

<i>Figure 8.</i>	Comparing ZOI value of autoclaved and not-autoclaved samples against MDR <i>P.aeruginosa</i> , (1.00E+08 CFU/mL). Double asterisks indicates significant different ( $p<0.05$ , $n=6$ ) between ZOI value of autoclaved samples and their not-autoclaved counterparts.....	57
<i>Figure 9.</i>	Comparing ZOI value of sterilized and non-sterilized samples against CA-MRSA (1.29E+08 CFU/mL). Double asterisks indicates significant difference ( $p<0.05$ , $n=6$ ) between ZOI value of autoclaved samples and their not-autoclaved counterparts. ....	57
<i>Figure 10.</i>	ATR-FTIR spectra of untreated Acticoat and PET. ....	59
<i>Figure 11.</i>	Comparing peeling energy of ethylene oxide sterilized samples with not sterilized samples. ....	60
<i>Figure 12.</i>	Comparing ZOI value of ethylene oxide- sterilized and not-sterilized samples against MDR <i>P.aeruginosa</i> , (8.00E+08 CFU/mL).....	61
<i>Figure 13.</i>	Comparing ZOI value of ethylene oxide- sterilized and not-sterilized samples against CA- MRSA, (5.5E+07 CFU/mL).....	61
<i>Figure 14.</i>	Weight increment of samples after hydrogel deposition. Double asterisks indicate significant difference ( $p<0.05$ , $n=10$ ) between the weight increment value of PET-PAM-10 and other values.....	62
<i>Figure 15.</i>	Comparing normalized swelling ratio in samples. No significant difference ( $p<0.05$ , $n=8$ ) was seen between the normalized swelling ratio value of PET-PAM-10 and other values.....	63
<i>Figure 16.</i>	Concentration of released TBO in 50% acetic acid. Double asterisks indicate significant difference ( $p<0.05$ , $n=3$ ) between the value of PET-PAM-10 and other values.....	66
<i>Figure 17.</i>	Comparing peeling energy of BDDAB loaded and not- loaded dressings, with PET dressing. Double asterisks indicate significant difference ( $p<0.05$ , $n=3$ ) between the peeling energy value of PET dressings and other values. ....	68

*Figure 18.* Weight of loaded biocide (mg) per samples weight (g). Double asterisks indicate significant difference ( $p < 0.05$ ,  $n = 15$ ) between the value of PET-PAM-10 and other values..... 70

*Figure 19.* Cumulative release from samples (mg/mL) ..... 72

*Figure 20.* Release of QASG1C12 versus time curves from hydrogel deposited samples. .... 73

*Figure 21.* Comparing ZOI diameter of samples against CA-MRSA. Double asterisks indicate significant difference ( $p < 0.05$ ,  $n = 6$ ) between the ZOI value of PET-PAM-10 samples and other values. .... 74

*Figure 22.* Comparing ZOI diameter of samples against MDR P.aeruginosa. Double asterisks indicate significant difference ( $p < 0.05$ ,  $n = 6$ ) between the ZOI value of PET-PAM-10 samples and other values. .... 75

## LIST OF SCHEMES

- Scheme 1.* First generation poly amidoamine (PAMAM) dendrimer: QASG1C12..... 37
- Scheme 2.* Images show inhibition zone of untreated silver dressings, and polyacrylamide treated dressings, against MDR *P. aeruginosa* and CA- MRSA. C and D refers to as Acticoat-PAM-10 and Acticoat-PAM-10-3.2Kpa respectively, while C' refers to Silverlon-PAM-10..... 46
- Scheme 3.* Observation of Acticoat dressing before and after coating their edges under the microscope. Left pictures are Acticoat-PAM-10 and right pictures are Acticoat sample. .... 48
- Scheme 4.* Images showing comparison between inhibition zones of edge coated samples and untreated samples. .... 49
- Scheme 5.* Released silver particles from treated and untreated Acticoat samples after 24 hours in DI water. Samples from left to right are: B: Acticoat, C: Acticoat-PAM-10, D: Acticoat-PAM-10-3.2Kpa..... 50
- Scheme 6.* Sample's color after immersion in TBO solution for 30 minutes. Samples from left to right are: PET, PET-PAM-10, PET-PAM-PAA-9-1, PET-PAM-PAA-7-3 and PET-PAM-PAA-5-5..... 64
- Scheme 7.* The released TBO from samples after placing them in 50% acetic acid for 1 hours (samples containing acrylic acid were 10× diluted for analyzing in UV-Spectroscopy). Samples from left to right are: PET, PET-PAM-10, PET-PAM-PAA-9-1, PET-PAM-PAA-7-3, and PET-PAM-PAA-5-5. .... 65
- Scheme 8.* Loaded Biocide on samples, Left to right: PET-PAM-10, PET-PAM-PAA-9-1, PET-PAM-PAA-7-3, PET-PAM-PAA-5-5..... 69
- Scheme 9.* Pictures on the left (1 and 3) show the ZOI diameter of samples on agar plate when samples were tested against CA- MRSA and pictures on the right (2 and 4) show the ZOI diameter of samples on agar plate when samples were tested against MDR *P.aeruginosa*. A: PET, B: PET-PAM-10, C: PET-PAM-PAA-9-1, D: PET-PAM-PAA-7-3, E: PET-PAM-PAA-5-5. (Sample E in picture 1 and 3

was wrinkled) Also ZOI of the dressings after removing them from agar are visible on picture 3 and 4. .... 76

## LIST OF ABBREVIATIONS:

<b>AgNPs</b>	Silver nano particles
<b>AM</b>	Acrylamide
<b>ATR-FTIR</b>	Attenuated total reflectance – fourier transform infrared Spectroscopy
<b>BDDAB</b>	Benzyl dodecyl dimethyl ammonium bromide
<b>CH</b>	Chlorhexidine
<b>CFU</b>	Colony-forming unit
<b>CMC</b>	Carboxymethylcellulose
<b>CT<sub>50</sub></b>	Cytotoxicity at which 50% of cells survive
<b>DI water</b>	Deionized water
<b>DMSO</b>	Dimethyl sulfoxide
<b><i>E.coli</i></b>	<i>Escherichia coli</i>
<b>IBU</b>	Ibuprofen
<b>ICP-OES</b>	Inductively coupled plasma optical emission Spectrometry
<b>IPN</b>	Interpenetrating network
<b>MBA</b>	N,N'-methylene bisacrylamide
<b>MRSA</b>	Methicillin-resistant <i>Staphylococcus aureus</i>
<b>MDR <i>P. aeruginosa</i></b>	Multi-drug resistant <i>Pseudomonas aeruginosa</i>
<b>MIC</b>	Minimal inhibitory concentration
<b>MTT</b>	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
<b>PAM</b>	Polyacrylamide
<b>PAM-10</b>	Polyacrylamide hydrogel deposited on dressings
<b>PAM-10-3.2Kpa</b>	Polyacrylamide hydrogel deposited on dressings Following 3.2 kpa pressures during preparation
<b>PBS</b>	Phosphate-buffered solution
<b>PBG</b>	Polycationic polybiguanide
<b>PET</b>	Poly(ethylene terephthalate) or polyester
<b>PET-PAM-PAA</b>	Polyacrylamide-co-acrylic acid hydrogel deposited on PET dressing
<b>PHMB</b>	Polyhexamethyl-biguanide

<b>PTFE</b>	Polytetrafluoroethylene
<b>PVA</b>	Polyvinyl alcohol
<b>QAs</b>	Quaternary ammonium salts
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<b>TBO</b>	Toluidine Blue O
<b>UV</b>	Ultraviolet
<b>WHO</b>	World Health Organization
<b>ZOI</b>	Zone of inhibition

*Chapter 1*  
*Introduction*

# CHAPTER 1. INTRODUCTION

## 1.1 Overview

According to data published in 2014 by the World Health Organization (WHO) approximately 270,000 people died in 2012 due to burn injuries. <sup>1</sup> Bacterial infection in wounds is a significant factor that can jeopardize healing and can even lead to a patient's death. <sup>2</sup> Burn injury causes a loss of the skin barrier, thus, providing easy access of underlying tissues for micro-organisms. <sup>3</sup> Necrotic tissues in the wound bed can act as a medium for micro-organism growth. Larger wound area increases the possibility of micro-organism transmission. <sup>4</sup>

An infection's hazardous rate depends on factors such as: the source of organism, transmission mode and a patient's susceptibility. <sup>4</sup> In order to decrease the possibility of wound infection, cleansing the wound and protecting it from the outside environment are recommended. <sup>5,6</sup>

Recently the use of topical wound dressings with antimicrobial ability has been increasing. <sup>7-9</sup> Silver, iodine, chlorhexidine and honey are among different antimicrobial agents that are used in these dressings. <sup>7</sup> Antimicrobial ability of a wound dressing is not the only criterion that makes a dressing favourable for clinical use. Experts reported a moist wound bed can improve and facilitate wound healing. <sup>10-12</sup> There are different dressings available on the market that are able to donate moisture or absorb excess exudate in the wound, depending on the wound requirement. <sup>8,9,13</sup>

In addition to antimicrobial ability and providing sufficient moisture for the wound bed, managing pain is also an important criterion in an ideal wound dressing. <sup>14</sup> According to Hollinworth and M. Collier <sup>15</sup> 81% of practitioners categorized dressing removals as the highest pain and trauma experienced in therapeutic procedures. Exudates, a proteinaceous fluid leaking from the wound bed, can penetrate through the structure of a dressing. Exudates

adhere to the dressing's fibre after drying. Furthermore the proteinaceous structure of exudates can chemically bond to the dressing (mostly hydrogen bonding).<sup>16</sup> Removal of the dressing adhered to the wound bed causes pain and trauma and can delay healing.<sup>17</sup>

In summary, an ideal dressing for a burn wound is required to be antimicrobial, pain free and able to control the wound moisture to facilitate healing. A dressing with a combination of these three criteria is still not available.<sup>14</sup>

Our work will concern the existing gap of knowledge which is the adhesion problem of current antimicrobial burn wound dressings. In this research, a hydrogel deposition method for improving the non-adherence properties of commercially available silver based wound dressings will be introduced. Furthermore, to create a versatile platform for the construction of non-adherent antibacterial wound dressings, a hydrogel surface will be created on pristine poly (ethylene terephthalate) (PET) fabric to serve as a reservoir for the loading of our newly synthesized poly(amidoamine) dendrimer based biocides. The composition of the hydrogel layer will be tuned to get a sustained release profile. This work will lay a foundation for the construction of non-adherent wound dressings loaded with various suitable topical biocides.

## **1.2 Burn injury**

According to the World Health Organization, a burn is a type of injury to skin or other body organs which can be caused by heat, friction, electricity, radiation, and chemicals. Injuries in the respiratory system due to inhaling smoke are also burn injuries.<sup>18</sup>

Burn wounds are globally the most serious forms of injury.<sup>2</sup> Patients with these types of injuries require immediate care to decrease the possibility of morbidity or mortality in them.<sup>2</sup> Approximately 270,000 people die each year due to burn incidents.<sup>18</sup> Burn injuries that are not fatal mostly come along with prolonged care and hospitalization. Burn injuries are sensitive and susceptible to future trauma.

Infection of a burn wound has been reported to be a common cause in most mortality during the first 5 days post burn.<sup>19-21</sup> The flora of the wound represents an opportunity for pathogens to colonize and infect the wound. Existing bacteria (found deep inside sweat glands and hair follicles that survived the initial heat) in a wound's substrate immediately after burning, are mostly gram positive such as *staphylococci*.<sup>2,22</sup> Gram negative bacteria and other types of pathogens from a patient's own body, hospital environment or health care provider's hand will also start to colonize the wound after a week.<sup>23</sup>

A wound is considered to be colonized when bacteria are present and start to multiply (below  $10^5$  bacteria per weight unit of tissue(g)). One step further is when this multiplication results in delayed healing and often along with resonance of pain. This situation is called critical colonization in which there is still no obvious host reaction. The wound infection starts when the bacteria and their multiplication are resulting in tissue damage, delayed healing and obvious host immunological reactions (at this stage each gram of tissue is associated with more than  $10^5$  bacteria).<sup>6,24-27</sup> When the wound faces with critical colonization it is time to consider implementing an antimicrobial dressing as a therapeutic procedure.<sup>28</sup>

### **1.3 Wound care and usage of wound dressing for burn wounds**

Therapeutic procedure after burn injuries are designed to avoid infection, tissue loss and support healing.<sup>29</sup> These procedures are required to be according to patient's expectations and as painless as possible.<sup>29</sup> In a non-specialist view, the first step of burn wound care is wound examination.<sup>5</sup> Depth, area and type of wound must be distinguished and the wound must be prepared by applying cold water, cleaning and covering with appropriate dressings.<sup>5</sup>

Skin acts as a barrier that protects internal tissues and organs against microorganisms and heat loss. Burn injury results in a disability of the skin to fulfill all or some of its tasks. Thus,

protecting the wounded area with a burn dressing can prevent microorganism invasion and evaporation loss from body.<sup>30</sup>

Choosing the best wound dressing for burn wounds depends on the availability of dressings and preference of clinicians. Selecting a proper burn dressing can affect the healing process of the wound. Alsbjörn *et al.* described some characteristics of a desirable burn wound dressing.<sup>5</sup> Easy application and removal of dressing (being as painless as possible) are one of the characteristics.<sup>5</sup> Other important characteristics include: dressing's ability to maintain the wound environment moist by absorbing excess exudate or donating moisture (as a dry or macerated wound bed prolongs the healing), as well as also protecting the wound from exterior pathogens and infections.<sup>5</sup> Thomas introduced flexibility and handling, fluid and odor absorbing, adhesive properties, and antimicrobial ability of a dressing as major factors to search for, in an applicable dressing.<sup>31</sup>

According to an online survey of specialists around the world done by Selig *et al.* in 2012, antimicrobial activity, non-adhesion, and absorbency are the most desirable criteria of an ideal burn wound dressing. Such a dressing with all these essential properties is still not available.<sup>14</sup> Hence, there is still room for scientists to improve existing dressings or introduce new dressings with the desired properties.

Nowadays several types of dressings are available on the market for burn treatments. Traditional burn dressings are combinations of paraffin-saturated gauze and an absorbent layer of cotton or wool.<sup>32</sup> However, these dressings are likely to adhere to the wound bed.<sup>33</sup> The current understanding of the wound healing process and improvements in technology have led to the introduction of a wide range of modern dressings. It is possible to categorize available dressings according to the characteristics needed for an ideal dressing, namely moisture absorption and retention, antimicrobial ability, and wound adherence.

### **1.3.1 Absorption and moisture retain dressings**

Studies have shown that the wound healing process can be accelerated in a moist environment.<sup>10-12</sup> A moist wound environment is not a wet wound, which is covered in fluid.

<sup>8</sup> An exudate is a fluid leaked from blood vessels that assists healing by creating moist environment in the wound and also providing nutrients needed for cells.<sup>34,35</sup> Exudates also enable tissue migration during healing process, aid separation of dead or harmed tissues and assist growing cells.

A change in exudate shows a change in healing of wound. The rate of exudate leakage depends on capillaries and pressure across the capillary walls. Depending on surface area of the wound the amount of exudates may be different. Some wounds, such as burn wounds, have higher exudate rates because they often have greater wound areas. Therefore, more exudate leakage is expected from them.<sup>35</sup> As exudate arrives to the surface of the wound, it can become contaminated with microorganisms or tissue debris.<sup>34</sup>

A proper dressing is required to be able to manage the moisture and exudate in the wound. This can be done by maintaining the moisture, absorbing excess exudates or by donating moisture to the wound.<sup>8</sup> Proper dressing can be chosen depending on the moisture condition of the wound.

There are different types of dressings which are labelled as having absorbency or moist retaining ability. These dressings are Tulle/paraffin gauze based, Alginate based and foam dressings (absorbent dressings), hydrocolloids and films (maintain level of moisture) and hydrogel dressings (moist donators).<sup>8,9,13</sup>

### **1.3.2 Antimicrobial wound dressings**

Human skin is vital for protecting the underneath tissues and organs against foreign hazards such as infections. Wound infection can delay the healing process or even lead to a patient's death.<sup>36,37</sup> Hence, controlling and reducing the risk of infection is critical. Paying attention to

hygiene especially in the case of burn patients reduces the risk of cross-contamination. Also, choosing proper agents for cleansing the wound in antimicrobial therapy is essential. <sup>6</sup>

Due to the extensive usage of antibiotics and increasing hazards of antibiotic resistance in bacteria, global strategies are to decrease consumption of these agents. <sup>38-40</sup> Recent developments lead to the introduction of antimicrobial dressings which inhibit or kill microorganisms. <sup>7</sup> Antimicrobial dressings offer advantages such as ease of usage and availability. They are mostly inexpensive compare to antibiotics and cause less risk of resistance when impregnated with antimicrobial agents other than antibiotic agents. <sup>7</sup> Silver, chlorhexidine, iodine, honey and polyhexamethyl-biguanide (PHMB) based dressings are some of the available antimicrobial dressings on the market. <sup>7,41-44</sup>

Techniques to confer antimicrobial activity to a textile depend on the antimicrobial agents. Pad-dry-cure, covalently bonding, cross-linking the agent to fabric/ fibres, and incorporating the antimicrobial agent during polymer formation in synthetic fibers are among conventional antimicrobial incorporating methods. <sup>45</sup>

### **1.3.2.1 Silver**

Among metal and metal salts, silver (Ag), due to its antimicrobial properties has by far a wide range of applications such as food, cosmetic and clothing production, water and air purification, clinical applications, and household products. <sup>45,46</sup> Among available antimicrobial burn wound dressings, several are impregnated with silver. Usage of silver for treating burn injuries has centuries of background. <sup>47-49</sup>

Silver antimicrobial dressings are often impregnated with Ag salt or nanoparticle metallic Ag. <sup>50</sup> The ionic form of silver ( $\text{Ag}^+$ ) is the biocidal agent in silver salts. <sup>49</sup> According to Xiu *et al.* <sup>51</sup> the antibacterial ability in silver nanoparticles are also mostly due to the release of ionic form of silver. Acticoat Flex, <sup>52</sup> Mepilex Ag, <sup>53</sup> Silverlon, <sup>54</sup> Actisorb, <sup>55</sup> Aquacel Ag <sup>56</sup> are some silver-impregnated commercially available dressings.

In the case of synthetic fibers, silver particles can be incorporated to fibers during manufacturing process.<sup>45</sup> (e.g. during polymer extrusion<sup>57</sup> or nanofiber electrospinning<sup>58-60</sup>) Trevira Bioactive, one of the commercially available polyester fabrics with silver containing fibers,<sup>45,61</sup> is manufactured by anchoring silver into polymer fibers during extrusion or blending process.<sup>45,61</sup>

Furthermore, silver nanoparticles have been impregnated on synthetic (polyester) and cellulosic (cotton) fabrics by padding a colloidal solution as finishing.<sup>62</sup> Chemical reduction process can be named as another silver incorporation method. For instance, Osório *et al.* reported successful Ag nanoparticle synthesis on the cotton and wool using a silver salt chemical reduction process.<sup>63</sup> Among silver incorporated fibres/fabrics with different methods, Sea Cell fibres that are made of seaweeds offer the advantage of permanent silver absorption due to their ability to absorb seawater minerals.<sup>64</sup>

### 1.3.2.2 Iodine

Iodine as an antimicrobial agent has been used from 19<sup>th</sup> century.<sup>65</sup> Molecular-iodine can penetrate through microorganisms and cause damage in their protein, fatty acid and nucleotides.<sup>66</sup> To overcome limitations of iodine - such as, irritation and staining while maintaining its antimicrobial ability - complexes with carriers such as Povidone and Cadexomer were introduced.<sup>67-69</sup> These complexes were made to provide a sustained release of iodine, make iodine more soluble and decrease the concentration of free molecular iodine in equilibrium.<sup>69</sup>

Due to universal fondness of silver based dressings for infected wounds, iodine wound dressings did not increased in number on the market.<sup>67</sup> Some available iodine dressings are: Inadine,<sup>70</sup> Iodosorb,<sup>71</sup> Iodoflex,<sup>72</sup> Iodozyme,<sup>73</sup>.

There are different techniques used in the literatures to incorporate iodine on the fibres or fabrics. Aubert-Viard *et al.* developed a chitosan coating on PET/Polypropylene textile using

a pad-dry-cure process. They loaded iodine/silver nitrate on these dressings by dipping them in the iodine/silver sulfate solution.<sup>74</sup> Mishra *et al.* prepared hydrogels using carboxymethyl cellulose (CMC), polyvinyl alcohol (PVA), polyacrylamide (PAM) and N,N'-methylene bisacrylamide (MBA) as a crosslinking agent. Povidone-iodine was loaded on their samples by swelling the dry hydrogel samples in povidone-iodine solution.<sup>75</sup>

### 1.3.2.3 Polybiguanides

Chlorhexidine (CH) and polyhexamethyl-biguanide (PHMB) are antimicrobial agents from polycationic polybiguanide (PBGs) family.<sup>76</sup> PHMB has been used as disinfectant, water treatment agent and in medicine.<sup>44,77,78</sup> Similar to PHMB, CH has also been used in medicine and also in dentistry.<sup>79,80</sup>

PHMB as finishing agent can bind to carboxylic groups of bleached or mercerized cellulose.<sup>44</sup> Moreover, if the cellulosic fabric is dyed with reactive dye (anionic), PHMB absorption on fabric will increase.<sup>44</sup> In addition, pre-treatment can improve exhaustion of the PHMB. As an example, Gao and Cranston found that, it is possible to increase the PHMB exhaustion on wool up to 5% by peroxymonosulfate and sulfite pre-treatment.<sup>81</sup>

Using resin or pad-dry-cure method have also been reported in literature for immobilizing PHMB: Payne and Yates introduced immobilization of antimicrobial agents such as quaternary ammonium salts (QAS) and biguanide to non-cellulosic fibers using a self-cross-linkable resin.<sup>82</sup> Virka and Ramswamy treated the surface of nonwoven fabrics for surgical gowns with different antimicrobial agents such as PHMB using a pad-dry-cure process.<sup>83</sup>

Also, Blackburn *et al.*, applied CH on cotton fabric by immersing it in aqueous solution of this compound and adjusting the pH to 7 via 0.1% sodium hydroxide (NaOH) and then heating at 40°C to allow sorption of CH. The binding was contributed to hydrogen bonding of biguanides and cellulose and also interaction between CH and carboxylic acid group of modified cellulose.<sup>84</sup>

Kendall Foam,<sup>85</sup> Suprasorb X+PHMB,<sup>86</sup> Telfa AMD,<sup>87</sup> are some available dressings that have PHMB as antimicrobial agent. Also, Bactigras,<sup>88</sup> is another available antimicrobial dressing impregnated with 0.5% chlorhexidine acetate.

#### **1.3.2.4 Honey**

Usage of honey in a wound dressing has been mentioned in ancient writings.<sup>89</sup> Medical honey (e.g. Monuka) can be incorporated to the dressing to reach antimicrobial ability. Hydrogen peroxide is the main compound causing honey's antimicrobial ability.<sup>90,91</sup>

Electrospinning has been reported to be among different methods for preparing a honey-based antimicrobial dressing. In a study by Maleki *et al.* a nano-fibrous wound dressing was produced using aqueous solution of honey and poly(vinyl alcohol) via electrospinning.<sup>92</sup> Bulman *et al.*, manufactured fibers of PVA and methylglyoxal by electrostatic spinning, Methylglyoxal used in their study is known to be non-peroxide bacteriostatic compound in Manuka honey.<sup>93</sup>

Also, Kuhade *et al.* developed a honey-hydrogel dressing. Initially they prepared an acrylamide hydrogel by mixing chitosan/alginate/gelatin in double distilled water for 24 hours and then added acrylamide (AM), MBA, ammonium persulphate plus honey to the mixture. Hydrogel was prepared by placing the samples at 60°C for 24 hours.<sup>94</sup> Activon Tulle,<sup>95</sup> L-mesitran,<sup>96</sup> TheraHoney,<sup>97</sup> are some of the available dressings with honey as antimicrobial agent in them.

#### **1.3.2.5 Advantages and disadvantages**

Antimicrobial agents used in available dressings have shown some inefficiency. For instance, In the case of silver: Bacteria such as *Salmonella*, *Escherichia coli* K-12 and *E.coli* O157:H7 have been reported to be resistance against silver compounds.<sup>47</sup> Also Tiot and Page compared silver and honey wound dressings for their cytotoxicity on fibroblast and

keratinocyte tissue cultures. Their results showed poor cell proliferation when silver dressing was tested against the cells compare to honey wound dressing.<sup>98</sup>

Other than silver, bacterial resistance to chlorhexidine and iodine has been reported in a few cases.<sup>68,99</sup> Moreover, when *in vitro* cytotoxicity of these agents was compared with each other in a study by Sanchez *et al.*; Chlorhexidine diacetate (cationic polybiguanide) and povidone-iodine showed to be lethal for canine fibroblast cells at their biocidal concentrations.<sup>100</sup> However, in another *in vivo* study on beagle dogs done by this group, it was mentioned that concentration of chlorhexidine diacetate which was toxic to fibroblast cells *in vitro* does not affect the wound healing.<sup>101</sup>

The antimicrobial dressings are planned to have a controlled release of antimicrobial agents. The sustained and slow release allows delivery of drugs at therapeutic dose, minimizing toxicity.<sup>102</sup> However, the available dressings in the market, mostly come along with some disadvantages.

For example, although reducing or managing infection depends on the nature of the incorporated agents, but patient may be sensitive to the antimicrobial dressing (iodine) or dressing can leave stain on the skin (silver).<sup>66,103-105</sup> Also, some antimicrobial agents such as PHMB are more effective when in direct contact with bacteria.<sup>104</sup> Even in some cases such as honey dressings, the treatment is diluted during time and dressing requires to be frequently changed to make sure that the antimicrobial efficiency maintains in the same level.<sup>90</sup> Furthermore, depending on the wound dressings' substrate they might adhere to wound and cause pain during removal.

Considering these disadvantages of most antimicrobial dressings and since antimicrobial ability of a dressing is not the only desirable property in an ideal dressing, choosing a proper dressing for burn wound is still a challenge. Among the available antimicrobial dressings for burn wounds, silver impregnated dressings, are the most common dressings.<sup>106,107</sup> The

possibility to improve these dressings to reach the characteristic of an ideal dressing would be of a great favor.

### **1.3.2.6 Evaluation of antimicrobial efficacy**

There are several methods available to evaluate antimicrobial efficacy of dressings. Agar diffusion assay, challenge tests or suspension tests such as Japanese Industrial Standard (JIS L 1902) or AATCC 100, preparing wound dressing extracts and studying the antimicrobial activity of the dressing's extract solution via microplate laser nephelometry and luminometric quantification of bacteria, are examples of available *in vitro* methods.<sup>108-111</sup> The results of the antimicrobial evaluation are affected by the selected method, the bacteria used for testing or by the solubility and the diffuse-ability of the antimicrobial agent.<sup>110</sup>

Diffusion assay or zone of inhibition assay which was standardize by Kirby *et al.* in 1956<sup>112</sup> could be considered as a simple and broadly accessible method to perform.<sup>28</sup> In this method, the antimicrobial disk (dressing) will be positioned on the surface of a bacteria-inoculated agar and will be incubated for 24 hours.<sup>28</sup> Existence or absence of bacterial growth around the antimicrobial disk shows the sensitivity of the tested organism to the antibacterial sample.<sup>113</sup> The results of diffusion method are reliant on the diffusion ability of the antimicrobial compound. Large molecules or bonded agents may not exhibit the same antimicrobial activity in disk diffusion assay as in the other tests such as challenge tests due to this matter.<sup>110</sup>

The challenge tests determine the efficacy of dressings by directly contacting them with the tested microorganisms.<sup>110</sup> In this method after direct contact and inoculation of fabric swatches with microorganisms for a certain amount of time, the antimicrobial agent is then neutralized and the antimicrobial ability is reported quantitatively or qualitatively.<sup>110,111,114</sup> This method is effortful and tedious especially for considerable number of samples.<sup>110</sup>

In addition of the above methods, as a last option when the final form of product is not applicable for toxicity testing, in order to more easily define the intended test substance, it is possible to extract it (using both polar and non-polar solvents depending on the test method and the nature of the dressing) following a technique reported in an ISO standard (DIN norm EN ISO 10993-12) and then determine the biological toxicity of the extracts.<sup>110,115-117</sup>

Wiegand *et al.* compared results of antimicrobial testing of silver dressings against *Staphylococcus aureus* and *Pseudomonas aeruginosa* when using 4 different test methods. The methods were agar diffusion test assay, challenge tests (JIS L 1902, AATCC 100), extraction-based methods (microplate laser nephelometry and luminescent quantification of bacteria). Extracts from silver dressings that were prepared according to DIN EN ISO 10993-12 were used for microplate laser nephelometry and luminescent quantification of bacterial methods. As mentioned by authors the agar diffusion method is not quantitative and the challenge tests overcome this problem, however the result of this test can be affected by absorption ability of samples. Authors suggested using several methods to determining the antimicrobial ability of samples.<sup>110</sup>

Bradshaw compared the antimicrobial ability of commercially available silver, honey and iodine based dressings against *E.coli*, *S. aureus* and *P. aeruginosa* by means of an agar diffusion assay. According to the author there was no significant difference between the antimicrobial agents' ability against the tested bacteria. The author also reported that individual dressings showed different antimicrobial ability against each strain which was due to dressing's structure affecting the compound's delivery ability.<sup>66</sup>

Castellano *et al.* evaluated antimicrobial effectiveness of commercial silver wound dressings using both agar diffusion assay and a quantification method against *E.coli*, *S. aureus*, *P. aeruginosa* and *Streptococcus faecali*. According to both methods they have reported higher bactericidal ability for dressings with higher silver concentrations.<sup>118</sup>

### 1.3.3 Wound adherence of burn wound dressings

When a dressing is removed from a wound, adherence of the dressing to the wound causes pain and also may damage regenerated skin.<sup>119</sup> Pain is one of the major issues that causes suffering in patients with burn injuries.<sup>120</sup> According to a study done by Loncar *et al.* the higher level of depression and anxiety in burn injured patients is related to the higher level of pain they experience in two weeks of their post burn.<sup>121</sup>

According to Choiniere *et al.* highest level of pain in burn injury patients was experienced during therapeutic procedure.<sup>122</sup> Dressing change is the pain associated therapeutic procedure and dressings must be changed every 2 to 3 days to minimize the possibility of infection.<sup>123</sup> Adherence of dressings to the wound can be due to the formation of a mechanical key between dressings and wound exudates when dressings are drying. It can also happen when the new tissue grows in the wound dressing structure.<sup>119,124</sup> Current dressings can be categorized based on their wound adherence into adherent, low-adherent, non-adherent and atraumatic.<sup>17</sup>

Adherent dressing as understandable from their names can adhere to any wound types. They are mostly cotton and gauze.<sup>17,124</sup> Removal of these types of dressings comes along with pain and damaging newly generated tissues.<sup>125</sup>

Low adherent dressings are majorly designed to allow exudates pass to a secondary dressing. These absorbent dressings reduce adherence to the wound and are useful for managing low to moderate exudate.<sup>126,127</sup> Some clinically available dressings are Melolin, Cutilin and Melolite.<sup>128</sup> Usage of absorbent dressing in the case of a dry wound may result in the dressing adherence.<sup>129</sup>

As mentioned both adherent and low-adherent dressings are not suitable dressings for the aim of pain prevention during dressing removal. Thus, non-adherent wound dressings were designed to prevent dehydration and adherence to wound by sustaining a moist gel layer over

the wound.<sup>17,124</sup> Petroleum-impregnated gauze, calcium alginate, hydrocolloid and hydrogels dressings are some of the current non-adherent dressings. Also, a recent type of dressing's classification called "Atraumatic" that cause no trauma to newly formed tissue or pre-skin while removal could be another option.<sup>130</sup> Soft-silicon based dressings that adhere to skin around the wound and not the wound itself are examples of atraumatic dressings.<sup>125,130</sup>

### **1.3.3.1 Advantages and disadvantages of non-adherent and atraumatic dressings**

Among the named non-adherent and atraumatic dressings, both have their own advantages and disadvantages. In the petroleum dressings the oily layer reduces the dressing's adherence and helps ease removal of the dressing; however, it reduces the concentration of oxygen around the wound and therefore it is not a favorable dressing.<sup>125</sup>

Calcium alginate based dressings are known for their hemostat ability in bleeding wound. They have absorptive capacity and can be used for highly exudate wounds. Gel forming property of alginate enables easy removal of dressing with less pain and can provide moist environment for healing.<sup>13,131</sup> However, Paddle-Ledinek mentioned that alginate dressing should be used with specific caution to the calcium alginate concentration due to its toxic effect in proliferation of keratinocyte.<sup>132</sup> Furthermore, absorptive properties of these dressing may result in future dehydration if applied over a dry or low exudate wound.<sup>133</sup>

Hydrocolloid dressings are carboxymethyl-cellulose gel forming dressings combined with elastomer and adhesives. These dressings have low moisture transmission rate. Although they can provide moist healing environment, they can also cause wound maceration in case of heavy exudate.<sup>133,134</sup>

Soft silicone dressings are newly introduced dressings, claiming to be atraumatic. Due to hydrophobic nature of silicon, these dressings adhere to the surrounding area of the wound and to the moist wound bed. These dressing are flexible and have the ability to adhere to uneven surfaces of skin causing adherence force to spread into larger area resulting in less

adherence force per millimeter square and therefore they cause less trauma while removal.

135,136

While atraumatic dressings have well-recognized advantages, according to Walker *et al.* soft silicon dressing (Mepilex Ag) has not shown to prevent bacterial growth in flat bacteria seeded agar although having silver as an antimicrobial agent. According to author's opinion, dressing's adhesive layer is the physical barrier preventing the antimicrobial silver from reaching bacteria surrounding the dressing.<sup>137</sup> Among the non-adherent dressing hydrogel can be chosen as a more promising material for burn wound dressing. Reasons are explained as continues.

### **1.3.3.2 Evaluation of adhesive dressings**

As explained before, dressing adherence to wound can cause pain during removal and may delay the healing process. The force utilized in the peeling of a dressing from the skin is the cause of trauma.<sup>138</sup> This force depends on the duration of the dressing contact with the wound, rate and angle of the peeling, temperature, humidity and it is also dependent on the dressing and the skin properties.<sup>138</sup> To design a proper dressing with low or no adherence to the wound, the peeling force that is required for removing the dressing must be considered. A standard peeling test can aid reaching this goal.

Many researchers measured the required peeling force for removing the dressing from healthy human volunteers. As an example, in a study by Waring *et al.* (supported by Molnlycke Health Care) the peeling force of removing two commercial foam dressings (Allevyn Adhesive: Smith & nephew and Mepilex Border: Monlycke healthcare) from the back of healthy volunteers was measured after 48 and 72 hours using a Universal Testing Machine (UTM). Humidity (50±5%) and temperature (21±1°C) were attuned 10 minutes before experiment. The dressings were then stripped at constant rate of 300 mm/min at 135° angle. In addition, the pain level among healthy volunteers during removal of dressing was

rated using a 0-3 (lest to highest) scale. Other methods such as analyzing the wound contacted side of dressings via SEM and analyzing concentration of protein peeled from healthy and peri-wound of patients through dressing removal were used to determine the least traumatic dressing. Mepilex Border dressing was reported to be atraumatic.<sup>139</sup>

Dykes *et al.* studied the required peeling force for removing five commercial adhesive dressings from 20 healthy volunteer's skin that were pre-stained (methylene blue was used as a stain for estimating damages incurred to the skin). The peeling was done at 135° and 25 mm/sec peeling rate, using a device built in their dermatology department.<sup>140</sup>

In another study, Karwoski and Plaut peeled the adhesive tapes from healthy human volunteers to study the factors concerning peeling force. The factors tested were peeling angle (90 to 180°), peeling rate (range from 100 to 10,000 mm/min), and dwell time (1 minute to 10 seconds). Instron 4505 and MTS were used for different parts of the test.<sup>138</sup>

An objection to these methods is using healthy human skin causes these methods to lack simulating effect of exudate drying or growth of newly generated tissue in a burn wound. Drying of exudate or growth of new tissue in the pores of the dressing obviously leads to increase in the peeling force.<sup>16</sup> Also *in vivo* evaluation on peri-wounded human volunteers is more comparable to the real situation but has drawbacks. The physiological state of patients and the type of wound may affect the reproducibility of these methods.<sup>141</sup>

In a study by Dong *et al.*, a machine was developed for measuring adherence of 2×2 cm<sup>2</sup> skin grafts to the surface of the wound in clamped mice. This machine had the advantage of reproducibility, the peeling procedure was conducted using a Holtzer-Cabot motor (at 2 rpm rotation rate) and the peeling force was recorded using a force transducer.<sup>142</sup> A concerning problem with this model is that using animals is of ethical apprehension. Furthermore, animals are mostly kept in conditions not similar to human and this can cause lack of applicability of these methods on humans.<sup>141</sup>

Some researchers considered designing *in vitro* methods to measure peeling force required for removal of a biomaterial. Bundy *et al.* described a peeling instrument for evaluating adhesion of soft tissues as an example wound dressing. The peel tester worked by peeling the soft tissue from a specimen (tissue adhesive) that was fixed in a holder at 90° while both tissue grip and specimen holder were moving at the same velocity.<sup>143</sup>

Although the device gave some flexibilities such as being portable and reducing the time loss during experiment,<sup>143</sup> since the biomaterial tissue should be kept fresh, the effect of wound desiccation and exudate dryness during time would have not been considered in that method.

In a study by Andrews and Kamyab an *in vitro* model for studying the adhesion of dressings was reported. To mimic different clinical situations of wounds, a gelatin model was used. After the wet gelatin was allowed to dry over certain times at 35°C, the peeling force required to peel the dressings was measured using Instron while removing the dressings at 180° and 100 mm/min peeling rate from the gelatin slab.<sup>16</sup>

This model is convenient and reproducible and has the advantage of mimicking the healing wound condition to some extents, due to drying of gelatin during experiment time.<sup>16,141</sup> Thus, among the explained models this model could aid in better differentiating the adherence level of different dressings.

#### **1.4 Hydrogel as a more promising material for burn wound dressings**

Considering disadvantages of previously mentioned dressings, according to Eisenbud *et al.* hydrogel has sufficient abilities to fulfill the role of an ideal dressing.<sup>144</sup> Hydrogel can provide a moist and healing environment which assists healing; it can protect the wound, it is easy to apply and remove, it is non-adhesive and it is cost effective.<sup>144</sup>

Hydrogels are three dimensional polymers that do not dissolve in water. They are capable of swelling and maintaining a large amount of water.<sup>145</sup> Hydrogels are used in contact lenses, drug delivery, wound dressing, diapers, tissue engineering and agricultures.<sup>146-150</sup> They are composed of hydrophilic moieties attached to a polymeric backbone, the polymers are attached together with crosslinkers that do not allow the 3D network to dissolve in water.<sup>145</sup>

Hydrogel dressings can prepare moist environment by donating moisture due to their water retaining ability.<sup>17,151</sup> To provide a satisfying condition for wound healing, dressings must sustain a suitable rate of water vapor transmission and water absorption capacity.<sup>152</sup> Controlling the wound fluid, preventing wound maceration and providing a moist environment aids healing.<sup>152</sup> A moist wound environment assists the wound healing process by cooling and pain-relieving, especially in burn wounds.<sup>17,151</sup>

Although hydrogels are categorized as occlusive dressings, they can absorb some wound exudate and allow water vapor permeation to a level that is comparable to a semipermeable membrane.<sup>153,154</sup> According to Liu *et al.* hydrophilic polymers such as hydrogels when swollen with water have higher gas permeability compared to their dried state.<sup>154</sup> Furthermore, hydrogels are easy to apply and remove. Removing hydrogels from wound does not cause trauma because of the moist environment they provide around the wound bed.<sup>134,144</sup>

#### **1.4.1 Hydrogel preparation**

Physical crosslinking, chemical cross linking, radiation cross linking and graft polymerization are different methods for producing hydrogel.<sup>146</sup> Hydrogel preparation depends on the existence of a monomer, an initiator and a cross-linker. Water and other aqueous solutions are needed to control polymerization heat. In order to remove unwanted side products and unreacted initiators, cross-linkers and monomers and impurities, the hydrogel needs to be washed.<sup>145</sup>

Among different methods of hydrogel preparation, irradiation is an easy to control process which can be done without adding cross-linkers and initiators (which are harmful to human body) and also the hydrogel can be formed in one step.<sup>155</sup>

Acrylamide and acrylic acid are among the most common monomers used to synthesize hydrogels.<sup>156</sup> Cross-linked polyacrylamides are nontoxic and non pH-dependent in their swelling ability.<sup>157,158</sup> Polyacrylamide hydrogels are being used for drug delivery purposes both *in vivo* and *in vitro*.<sup>159,160</sup> In addition acrylamide hydrogel has been used in our group earlier and showed reduction of the PET dressing adherence to the simulated wound model.

In one study, polyacrylamide hydrogel was prepared by immobilizing acrylamide on to PET surface via interpenetrating polymer network method. The resulted dressing was significantly less adherent compare to the pristine PET. Antimicrobial agents such as silver nanoparticles and *N*-chloramine were also immobilized to that dressing to enhance the antibacterial functionality of that dressing.<sup>161</sup> In another study, a layer of poly(acrylamide) hydrogel was deposited on poly(ethylene terephthalate) via UV radiation method to decrease the peeling energy needed for the removal of this dressing from a gelatine cast.<sup>162</sup>

#### **1.4.2 Hydrogel in drug delivery**

Among the different applications of hydrogel, much research has been done on drug delivery properties of hydrogels.<sup>163-165</sup> The porous structure of hydrogel allows drug incorporation; furthermore, biocompatibility of hydrogel is an important characteristic in drug delivery allowing successful functioning in living systems.<sup>159</sup> Loading drug into hydrogel can be done both during hydrogel formation (in-situ) and also by swelling the previously formed hydrogel in a drug solution for sufficient time to reach equilibrium of drug loading (post loading).<sup>166</sup> Among accessible literatures both in-situ loading and post loading methods have been used for drug loading in polyacrylamide hydrogels.<sup>167,168</sup> The later method is more advantageous due to the possibility of drug being affected by the polymerization procedure.<sup>166</sup>

In the post loading method, drug uptake depends mostly on the diffusion and the release of drug is determined by gel swelling and diffusion.<sup>169</sup> In the in-situ loading method, the drug and polymer solution will be mixed and the drug encapsulation and hydrogel network will be formed simultaneously. The drug release will be dependent on different factors such as drug-polymer interaction, degradation of covalent bonds in the system, hydrogel swelling and diffusion.<sup>169</sup>

The post loading method has been previously used in our group for loading novel *N*-chloramine and quaternary ammonium moieties into polyacrylamide hydrogel. Loading was done by placing 2×2 cm<sup>2</sup> samples in 2 mL of compound/PBS (pH=7.4, 0.1 M) solution at 32°C for 90 minutes and shaking it at 120 rpm. The release of biocide was measured using UV-spectroscopy at 485 nm and Fickian transport was reported for all samples.<sup>141</sup>

Upon swelling the gel in aqueous solution the drug can be released in the surrounding environment.<sup>170</sup> Hydrogel pore size, hydrophobicity, degradability, drug concentration and incorporation of drug and hydrogel are factors that can affect release from hydrogel network.<sup>171</sup> It is possible to tune hydrogel and reach a sustained release over an extended period.<sup>170</sup>

#### **1.4.2.1 Tuning hydrogel to achieve controlled release**

Different strategies to reach a sustained release from hydrogels are based on the interaction of hydrogel network and the loaded drug or changes in the diffusive barrier of hydrogel.<sup>165</sup> Interaction of the drug and the hydrogel can be physical (ionic interaction) or chemical (covalent bonding).<sup>165</sup> Change in ionic strength of hydrogel, pH or temperature of the environment can cause change in swelling of an ionic polymer and change the drug-hydrogel interaction.<sup>172</sup> It is possible to control the drug release via the polymer cleavage rate (enzymatic or chemically) after covalently bonding with polymer.<sup>165</sup>

In addition of changing the hydrogel-drug interactions, the structure of hydrogel can also be modified to control the diffusion rate.<sup>165</sup> Increasing the cross-linking density is a possible

strategy. However, the high cross-linking density may lead to unwanted properties.<sup>165</sup> Therefore, combination of both methods can be useful to reach the preferred release behavior.

Hydrogels can respond to several controlled release systems such as: swelling controlled, diffusion controlled, chemically controlled and environmentally responsive systems.<sup>173</sup> In a non-degradable system (e.g. diffusion and swelling release systems), drug release is reliant on the concentration gradient.<sup>174</sup>

In the case of a diffusion controlled system, the delivery device does not swell or change in size and the drug diffuses due to the difference in concentration between interior and the exterior environments of the hydrogel matrix.<sup>174</sup> In a swelling controlled release system, after a polymer come into contact with water or the proper fluid, it will start swelling. The drug will then start diffusing out of the area between swollen chains due to the concentration gradient.<sup>173</sup>

According to Ritger and Peppas, a swelling system can be described as a Fickian and non-Fickian release mechanism using the equation " $M_t/M_\infty=kt^n$ " (page 24 of reference<sup>175</sup>). Here, ' $M_t/M_\infty$ ' is the released fraction of drug during time t; 'k' is kinetic constant integrating geometric and structural characteristic of delivery system; and 'n' is the diffusion exponent suggesting the type of drug transport phenomena.<sup>175</sup> When a thin film is considered,  $n \leq 0.5$  shows a controlled diffusion mechanism (Fickian);  $0.5 < n < 1$  is related to anomalous or non-Fickian transport; and  $n=1$  is related to controlled swelling.<sup>141</sup>

Several studies have considered the concept of swelling controlled release of hydrogel to attain the goal of drug delivery. As an example, Risbud and Bhonde synthesized and characterized a polyacrylamide-chitosan hydrogel.<sup>159</sup> Although the drug loading method was not explained in detail, the control release properties of their hydrogel was studied by suspending amoxicillin loaded samples in a 50 mL pH=7.4 PBS at 37 degree centigrade.<sup>159</sup> Using a spectrophotometric assay, their measurements showed an initial zero-order release,

which was explained by pointing out the fast initial swelling of the hydrogel.<sup>159</sup> The release rate was decreased due to low swelling rate between 6 to 24 hours.<sup>159</sup> The continuous release after swelling ends was due to diffusion of drug entrapped in the polymer network.<sup>159</sup> Another example is investigation of the drug release properties of a thermally polymerized semi-interpenetrating network (semi-IPN) by Reddy *et al.*<sup>176</sup> The drug release in PBS (pH=7.4) at 32°C showed faster release at initial stages due to rapid swelling of samples.<sup>176</sup>

Although it is possible to attain zero-order release in the initial stages of release procedure due to swelling of the hydrogel, in most cases the release will slow down due to lowering or ending of hydrogel swelling. A hydrogel which can be triggered to swell by changes in the surrounding environment is considered “responsive” and can further aid the drug delivery.<sup>174</sup>

Different factors that affect the swelling ability of hydrogels include cross-linking ratio, pH, temperature, and ionic strength.<sup>174</sup> A higher cross-linker-to-monomer ratio will result in less swelling of the hydrogel network.<sup>177</sup> For instance, Soppiranth & Aminabhavi found that increasing the glutaraldehyde cross-linker in their guar gum and polyacrylamide hydrogel deviated the transport of water from a Fickian mechanism to a non-Fickian mechanism.<sup>168</sup>

In addition to cross-linking agent, changing the ionic strength or pH can affect the swelling of pH or ionic strength sensitive hydrogels.<sup>177</sup> In a study by Sadeghi and Hosseinzadeh, poly(sodium acrylate-*co*-acrylamide) hydrogel was prepared by hydrolysis reaction of starch and poly(acrylonitrile). Ibuprofen (IBU) was loaded on the superabsorbent hydrogels by immersing the powdered samples in alkaline solution of this drug at 0°C for 25 hours. Samples were then dried in vacuum oven at 37 °C. The *in vitro* release was done by incubating the loaded hydrogels in dialysis bag in 50 mL pH=1.2 or 7.4 buffer solution at 37 °C. The UV-spectroscopy measurements showed an increase in the drug release from hydrogels after increasing pH from 1.2 to 7.4. This was explained by pointing out the higher

repulsion between carboxylic acid groups in pH=7.4 and decrease in gel swelling in pH=1.2 which causes lowering the release of IBU.<sup>178</sup>

Also, Thakur *et al.* synthesized poly(acrylamide-*co*-acrylic acid) hydrogel via a radiation method using MBA as cross-linker. The repulsion between carboxyl groups in pH=7.4 at higher acrylic acid to acrylamide monomer ratio led to the creation of larger pores in hydrogel. Authors then investigated the effect of hydrogel composition and degree of cross-linking on the release of gentamicin sulfate as a model drug in phosphate buffer (pH=7.4). A deviate from anomalous diffusion to Fickian diffusion release behavior was reported at higher crosslinking ratio or concentration of acrylic acid.<sup>179</sup>

It is worthy of note to mention that changes in the polarity of hydrogel structure also help regulate the drug release. For instances, Sripriya and colleagues developed a bilayer dressing that has polarity controlled release. The anionic structure of dressing in pH=7.4 acted as a reservoir, holding Ciprofloxacin which is cationic in pH=7.4. The anionic binding regulated the release of drug from the dressing structure up to 5 days.<sup>180</sup> In summary, depending on the intended drug polarity and release environment pH it is possible to play with hydrogel structure and reach the proper loading and release behavior.

***Chapter 2***  
***Hypothesis and Objectives***

## CHAPTER 2. HYPOTHESIS AND OBJECTIVES

Burn injuries are among the traumatic forms of injuries. Infection of the burn wound post-burn can lead to mortality in patients.<sup>2</sup> To decrease the possibility of wound infection, it is recommended to cleanse the wound and protect it from the outside environment.<sup>5,6</sup> Recently, using topical wound dressings with antimicrobial ability has been increasing.<sup>7-9</sup>

Silver-impregnated dressings are common for burn wounds treatment and using silver as the antimicrobial agent has centuries of background.<sup>47-49,181</sup> Despite the commercial success of this antimicrobial agent, there are reports of the cytotoxicity and bacterial resistance of this agent or its derivative.<sup>181-186</sup>

Development of *E. coli* strain resistance to silver nitrate at the minimum inhibitory concentration (MIC) value (2-4 mg Ag<sup>+</sup>/L) or lower than MIC value of cationic silver is an examples.<sup>181,187</sup> Also silver nitrate was reported to be toxic on mouse fibroblast cells.<sup>183</sup> Thus, development and manifestation of new antimicrobial agents are also a requirement. It is possible to state that hundreds and thousands of polymeric compounds have been produced and verified for their antimicrobial ability; until now and very few of them are accepted.<sup>188</sup>

In addition to antimicrobial ability, providing sufficient moisture for the wound bed and managing pain are also important properties in an ideal wound dressing.<sup>14</sup> Studies have shown wound healing process can be accelerated in a moist environment.<sup>10-12</sup> As mentioned, an ideal dressing must also be able to manage pain. Removing a dressing adhered to the wound causes pain and also may damage regenerated skin.<sup>119</sup> Pain is one of the major issues that causes suffering and can lead to depression in patients with burn injuries.<sup>120,121</sup> A dressing with a combination of these three criteria is still not available.<sup>14</sup>

This dissertation aimed to fill the current lack of ideal burn wound dressings by 1. Decreasing the adherence of commercial silver based dressings and 2. Introducing a wound dressing with properties of an ideal dressing (non-adherent, absorbent, antimicrobial). A new

positively charged quaternary ammonium based dendrimer biocide compound prepared in our lab with promising antimicrobial activity and low cytotoxicity at the MIC concentration (MIC for MDR *P.aeruginosa* was lower than 31.73 µg/mL and CT50 concentration was higher than 52.88 µg/mL) was used as the antimicrobial agent for the second aim.

Two hypotheses were proposed in this dissertation. For the first aim, it was hypothesis that the deposition of polyacrylamide (PAM) layer on the commercial silver-based dressings will improve the non-adherent properties of these dressings without compromising their other properties. To achieve the second aim, it was hypothesized that copolymerization of acrylic acid and acrylamide will aid better loading and release of our positive dendrimer biocide.

The dissertation will be continued in two parts. Each part will be the attempts to test each of these hypotheses. In the first part the process of polyacrylamide deposition on silver based (two dressings tested were a Nanocrystalline dressing (Acticoat) and a silver plated dressing (Silverlon)) and PET dressings via UV-radiation grafting method will be explained. Resulting dressings will be tested for their adherence to the simulated exudate model (gelatine cast). Next, the effect of the PAM loading will be studied on the antimicrobial ability and silver release of the dressings via respectively disk diffusion test and ICP-OES measurement. The toxicity of treated and not treated samples will be then investigated on fibroblast and keratinocyte cells.

In the second part, the deposition of poly(acrylamide-co-acrylic acid) hydrogel with different monomer ratio on PET fabrics using similar UV-radiation grafting method will be explained. After measuring the concentration of carboxyl groups and peeling energy of the samples, the positive dendrimer biocide will be loaded on the samples and the release behaviour of samples will be studied via an immersion method. The biocidal ability of loaded dressings will be investigated and the best monomer ratio to achieve a dressing with properties of an ideal dressing will be determined.

*Chapter 3*  
*Materials and Experiments*

## CHAPTER 3. MATERIALS AND EXPERIMENTS

### 3.1 Materials

Acticoat Flex 3 was purchased from Smith & Nephew, Hull, UK. Silverlon was purchased from Argentum Medical, LLC, Geneva, IL/United States. Woven plain PET, no. 777H was purchased from Test Fabrics, Inc., West Pittston, PA/United States. Acrylamide (AM), acrylic acid, N, N'-methylenebisacrylamide (MBA) and Toluidine Blue O (TBO) were purchased from Sigma-Aldrich, Oakville, ON/Canada. Gelatine Type A was purchased from Fisher Scientific, Ottawa, ON/Canada.

Community-associated (CA)-MRSA #40065 and multi-drug resistant (MDR) *P. aeruginosa* #73104 were used as the model microorganism to challenge all the biocides. Both were clinical strains obtained from the Canadian Ward Surveillance (CANWARD) study evaluating antimicrobial resistance in Canadian hospitals, [www.canr.ca](http://www.canr.ca). ATCC-PCS-201 neonatal human dermal fibroblast was purchased from Cedarlane Corporation, Canada. Tryptone Soya agar was purchased from OXOID LTD., England. 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) used for determining cell viability was purchased from Sigma-Aldrich, Oakville, ON/Canada.

### 3.2 Experimental part 1

#### 3.2.1 Sample preparation and polyacrylamide (PAM) hydrogel deposition

Pieces of  $6 \times 14 \text{ cm}^2$  from silver based wound dressings (Acticoat Flex 3 and Silverlon) and also PET were treated with  $\text{O}_2$  plasma at a flow rate of 24-26 sccm for 20 minutes. Subsequently fabrics were treated with 5 mL monomer solution drop wisely.<sup>162</sup> The monomer solution was a mixture of 1.38 mol/L acrylamide (AM) and 0.013 mol/L N, N'-methylenebisacrylamide (MBA) in distilled water; in other words, containing a 9.8% w/v AM monomer and a 0.2% w/v MBA cross-linker<sup>162</sup> were dissolved in distilled water and a 10%

w/v polyacrylamide monomer solution was prepared (PAM-10). The solution was deoxygenated by bubbling nitrogen gas. Fabrics were then sandwiched between two glass plates and exposed to UV irradiation (Intelli-ray 400, Uvitron International, United States) with  $48.5 \pm 3.6$  mW/cm<sup>2</sup> intensity for 15 minutes. Samples were then rinsed with distilled water and the un-grafted monomer was removed by shaking the samples for two hours in reciprocal shaking bath at 65 °C and 150 RPM (The washing solution turned dark, which was due to release of silver in the washing temperature). Samples were then dried in oven at 105°C for 30 minutes and stored in desiccator for 24 hours.

Other than PAM-10 different concentrations of monomer solution (i.e. 5% w/v (PAM-5), 8% w/v (PAM-8)) while maintaining the cross-linker/monomer ratio constant, were used to treat PET samples. This experiment was done to find the optimum point with the lowest swelling ratio and energy needed to peel off gelatine samples.

In order to test the effect of hydrogel thickness on the properties of wound dressing similar treatment was done on another batch of samples while placing two pressure blocks of 1016.735 (PAM-10-2Kpa), 1626.778197 (PAM-10-3.2Kpa) and 2643.515 (PAM-10-5.2Kpa) Pascal on both sides of upper glass slide during the UV exposure procedure.

### **3.2.2 Swelling ratio:**

In order to calculate the swelling ratio, dressings were soaked in deionized water for 5 minutes and then centrifuged at 2800 rpm for 1 minute and 30 seconds to remove excess water while a cotton ball was placed underneath them. The test was done in triplicate and the swelling ratios of samples were calculated according to:

**Equation 1- Swelling ratio (%)**

$$Swelling\ ratio(\%) = \frac{(W_2 - W_1)}{W_1} \times 100$$

W<sub>1</sub>: Dry sample weight, W<sub>2</sub>: Weight of swollen sample

### 3.2.3 Peeling force test

The Instron 5956 machine was used for peeling force test.  $3 \times 14 \text{ cm}^2$  pieces from treated and not treated dressings were immersed in deionized (DI) water. Both Acticoat and Silverlon dressings were allowed to absorb water up to  $5 \pm 0.1$  and  $3 \pm 0.1$  times their weight respectively and PAM treated PET samples were removed from DI water after 5 minutes and centrifuged for 90 seconds to remove the excess water from them while a cotton ball was placed underneath them. Samples were then spread and the poly-tetrafluoroethylene (PTFE) frames with  $60 \times 15 \text{ mm}^2$  cavity were placed over them.

To simulate wound exudates adherence to the dressing a 40 wt% gelatine type A was prepared with 70 °C deionized water and then transferred into PTFE moulds. Samples were subsequently placed in the incubator at 32°C (temperature of human skin) and 75% humidity (mimicking a moist wound environment) for 24 hours.

The peeling force test was done by peeling the gelatine off from samples at a rate which was constant on 100 (mm/min) with peeling angle of 180°. The peeling test was done in triplicate and the average load needed to remove the gelatine from dressings between 20 to 80 mm was recorded.

### 3.2.4 Disk Diffusion test

The effect of polyacrylamide layer deposition on biocidal ability of samples was investigated by means of an agar diffusion susceptibility test. Both gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and gram-negative bacteria *Pseudomonas aeruginosa* (MDR *P. aeruginosa*) were chosen for the test, since they appear to infect the wound mostly.<sup>2,189,190</sup>

Both CA-MRSA (40065) and MDR *P. aeruginosa* (73104) isolates were streaked on Tryptone Soya agar plates and incubated for 18 h at 37°C. The growth suspension was prepared by adjusting the turbidity according to 0.5 McFarland standards. The resulting

concentrations were respectively 1.00E+08 and 1.29E+08 (CFU/mL) for *P.aeruginosa* and MRSA. A sterile cotton swab was dipped into standard suspension and streaked on the surface of Soya Tryptone Agar. The surface of agar plates were evenly inoculated by repeating the streaking two more times, each time the plate was rotated approximately 60°.

Round disks of 0.7 cm in diameter from both Acticoat and Silverlon samples were cut. They were pre-soaked with 1mL PBS and placed on the agar. Testing was done in triplicate. The samples were then incubated for 18 hours at 37°C. The diameter of the ZOI was measured in two directions using Vernier callipers, and the average data points were reported.

### **3.2.5 Suspension test**

As a confirmation to disk diffusion results a suspension antimicrobial test was also conducted to compare the antibacterial activity of dressings before and after the hydrogel deposition<sup>191,192</sup>. PAM hydrogel was grafted onto pre-cut dressing samples (1×1 cm<sup>2</sup>) (thereby coating the edge). Each 1×1 cm<sup>2</sup> sample was soaked in a vial containing 5 mL bacterial suspension (CA-MRSA, MDR *P. aeruginosa*) with a bacterial concentration of 10<sup>6</sup> CFU/mL.

The vials with bacterial suspension and dressings were placed inside the incubator at 37°C for 30min, 1h, 2h and 24 hours. At the end of each contact time, 100 µL aliquot from each sample was withdrawn and the aliquots were neutralized with 0.9 mL of D/E broth (1:9 ratio of test solution to D/E broth ratio) and colonies in the aliquots were enumerated by plating these bacteria on Tryptone Soya agar plates.

### **3.2.6 Silver release test**

To investigate the effect of polyacrylamide non-adherent layer on the release of silver ion from the Acticoat dressings, the concentration of released Ag<sup>+</sup> from the dressings were determined using inductively coupled plasma optical emission spectrometry (ICP-OES)<sup>50</sup>. The treated and untreated Acticoat dressings (2.5×2.5 cm<sup>2</sup>, 0.075 g) were first transferred into sterile vials. Deionized water was used as the media for silver release in a 1:100 (w/w) ratio.

The vials were capped and placed in an incubator at 32°C from 30 minutes, 2 hours and 48 hours (as the maximum duration of dressing application is 2-3 days). Each condition was done in triplicate. After each predetermined time interval, solutions were smoothly mixed by pipetting up and down, and one mL aliquots were withdrawn from each vial and kept in Eppendorf tubes. After each sampling, the vials were topped up with fresh deionized water to keep the volume constant. Prior to the ICP-OES analysis, the insoluble Ag species were removed from suspensions by centrifuging at 14,000 rpm for 10 minutes. Each 1 mL aliquot was diluted into 4 mL (10%) HNO<sub>3</sub> before being injected into ICP-OES (Varian 725ES ICP-OES instrument) for analysis (mean± standard deviation of the release in each time interval was reported).

### **3.2.7 Cytotoxicity test**

We evaluated the biological effect of depositing polyacrylamide non-adherent layer on dressings by applying an *in vitro* cytotoxicity test on fibroblast cells (ACTT-PCS-201 neonatal human dermal fibroblast) and Keratinocyte cells [HaCat].

Fibroblast cells are common cells in connective tissues. They are mostly known for their role in wound healing.<sup>193</sup> When tissue is injured, these cells start to migrate and deposit collagen in the damaged site making the healing process easier.<sup>194</sup> Keratinocyte cells are the cells existing in the outer layer of skin. They separate the organism from its environment.<sup>195</sup> On the other hand they “are known to kill living microbes”.<sup>196</sup> The purpose of using a topical antimicrobial dressing is to reduce infection; ideally burn dressing should not compromise the healing process.<sup>197</sup> Since the viability of fibroblast and keratinocyte cells are important in the healing process, cytotoxicity of the wound dressing on these cells are worthy of note.

0.7 cm diameter round disks were steam sterilized by autoclaving at 121.1°C for 30 minutes. The dressings were then pre-soaked in 0.4 mL of saline/DI water for 10 minutes at 37°C.

Cells were cultured at density of  $1-1.5 \times 10^5$  (cell/mL) in the 24 well-plates. Experimental treatments were applied after reaching 90% confluence. Each condition was done quadruplicate (4 wells /sample). Cytotoxicity testing was performed on treated and untreated Acticoat and Silverlon dressings in saline and water as delivery media.

1.1 mL of the culture medium was added to each wells and the dressings together with 0.4 mL saline or DI water were then added to each well. A solution of 0.4 mL saline or DI water plus 1.1 mL culture medium without dressing was regarded as a control condition. Afterwards, the cells were incubated for 24 hours at 37 °C. Viability of the cells was determined using MTT assay after removal of the dressings.

Each well received 500  $\mu$ L of 1:10 (v/v) MTT and cell's medium solution. Subsequently after 2 hours incubation at 37°C, the culture medium with the MTT solution were aspirated and replaced by 500  $\mu$ L dimethyl sulfoxide (DMSO). Finally 100  $\mu$ L aliquots from each well (in triplicate) were transferred to 96-well plates and viability of cells was evaluated using spectrophotometer at 570 nm wavelengths. (PowerWave™ XS2 Microplate Spectrophotometer, BioTek Instruments Inc., Canada)

### **3.3 Experimental part 2**

#### **3.3.1 Sample preparation**

In the second part of this project an electrolyte hydrogel layer was introduced to improve the loading and release of newly synthesized dendrimer biocide. Solutions containing different monomer ratio (M) of acrylamide to acrylic acid were prepared according to Table 1. The solutions were deoxidized via nitrogen bubbling. Hydrogel deposition procedure was done according to explained method on deposition of PAM-10 on PET dressings. After scratching the surface of  $6 \times 14$  cm<sup>2</sup> PET fabrics via O<sub>2</sub> plasma for 20 minutes at 24-26(sccm), samples were treated via 5 mL monomer solution drop-wisely. Next they were sandwiched between glass slides and UV-radiated with  $48.5 \pm 3.6$  mW/cm<sup>2</sup> intensity for 15 minutes in case of PET-

PAM-10 and 30 minutes in case of polyacrylamide-*co*-acrylic acid (PET-PAM-PAA) samples. Next, samples were rinsed with DI water and the un-grafted monomers were removed by placing the samples in shaking bath at 65 °C and 150 rpm for 2h. Afterwards, samples were dried in oven at 105 °C and then stored in desiccator for future usage.

**Table 1- Acrylamide/acrylic acid monomer preparation**

<i>Samples</i>	<i>Acrylamide (M)</i>	<i>Acrylic acid (M)</i>	<i>N,N'-methylenebisacrylamide(M)</i>	<i>Cross-linker/monomer ratio (M/M)</i>	<i>Monomer/monomer ratio (M/M)</i>
PET-PAM-PAA-9-1	1.2408	0.1359	0.01297	0.0094	9.1240
PET-PAM-PAA-7-3	0.9594	0.4135	0.01297	0.0094	2.3200
PET-PAM-PAA-5-5	0.6893	0.6938	0.01297	0.0094	0.9935

### 3.3.2 Weight increment and swelling ratio

Hydrogel deposition on samples was calculated according to Equation 2 where  $W_1$  is weight of untreated PET and  $W_2$  is weight of samples after hydrogel deposition.

**Equation 2- Weight increment (%) of samples after hydrogel deposition**

$$Weight\ increment(\%) = \frac{(W_2 - W_1)}{W_1} \times 100$$

Swelling ratio of samples was evaluated in PBS (pH=7.4, 0.1 M) via centrifuge method. 2\*14 cm<sup>2</sup> samples were placed in a beaker of PBS for 5 minutes and then centrifuged at 2800 rpm for 90 seconds while a cotton ball was placed underneath them. The swelling ratio was calculated according to the previously stated Equation 1. Ten and eight replicates were respectively performed for weight increment and swelling ratio test and mean ± standard deviation were reported.

### 3.3.3 Determination of carboxylic acid content on samples

A staining method with Toluidine Blue O (TBO) was used to determine the concentration of carboxyl groups in samples. 1x1cm<sup>2</sup> samples were cut and immersed in 10 mL of 0.5 mM

TBO (pH adjusted to 10 via NaOH solution) solutions for 30 minutes at room temperature while shaking. Samples were then rinsed using NaOH solution (pH=10) and DI water while vortexing to remove the free TBO. After drying the samples in 105°C, the captured TBOs were released in 30 mL of 50% acetic acid solution by immersing the samples for 1 hour until getting a blue solution. The absorbance of the TBO containing solutions was measured via UV-spectrophotometer (Ultrospec 4300 pro, Biochrom, England) at 630 nm and the concentration of TBO in each solution was calculated using the calibration curve of TBO in 50% acetic acid that was plotted prior to the experiment. The experiment was done in triplicate and mean± standard deviation is presented.

#### **3.3.4 Peeling force test**

Peeling force test was done similar to the experimental part 1. Instron 5956 machine was used for peeling force test. Pieces of 3×14 cm<sup>2</sup> from samples were immersed in deionized (DI) water for 5 minutes and the excess water was removed from the samples after centrifuging them for 90 seconds at 2800 rpm. Samples were then spread and after placing PTFE frame, the 40% gelatine solution was poured on them. After 24 hours of incubation at 32°C and 75% the gelatine cast was ripped off from samples at 100 (mm/min) constant rate and 180° peeling angle and the peeling energy was recorded. Test was done in triplicate and the average load between 20 to 80 mm was recorded.

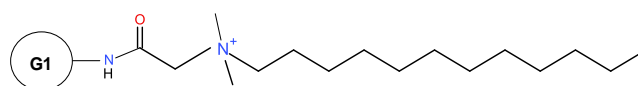
To study the effect of loading the quaternary ammonium based biocide on the peeling energy and adherence samples, Benzylododecyldimethyl ammonium bromide (BDDAB) was chosen as a model drug. Loading the model drug on samples was done by a similar method to biocide loading. Samples were immersed in 10 mL of 8 mg/mL BDDAB solution and shaken for 90 minutes at room temperature; samples were then dried at 105°C and stored in desiccator to reach a constant weight. The peeling test was repeated on the drug loaded

samples in triplicate and peeling force at 100 (mm/min) constant rate and 180° peeling angle was recorded.

### 3.3.5 Synthesizing a new biocides

Recently we have synthesized a new poly(amidoamine) dendrimer based biocide (

Scheme 1) whose MICs against multidrug resistant MDR *P. aeruginosa* ( $< 31.73 \mu\text{g/mL}$ ) is lower than its CT50 to the skin cell fibroblast ( $> 52.88 \mu\text{g/mL}$  biocide concentration), holding promise to be used for wound infection control. The biocide synthesized in our lab was long chain quaternary ammonium biocides with first generation poly amidoamine (PAMAM) dendrimers (QASG1C12).



Scheme 1- First generation poly amidoamine (PAMAM) dendrimer: QASG1C12

### 3.3.6 Loading compound QASG1C12 on PET hydrogels

A post-loading method was used to load the biocides on samples. 2 mL of 0.002137M or in other words 8 mg/mL of biocide-PBS solution (QASG1C12-PBS (0.1M and pH=7.4)) was used for loading. Dried pieces of  $2 \times 2 \text{ cm}^2$  samples were cut and placed in vials containing the biocide-PBS solution. Loading was done while shaking for 90 minutes at room temperature. Samples were then dried in  $105^\circ\text{C}$  and placed in desiccator to reach constant weight. Weights of loaded biocides were measured by subtracting the weight of samples before and after loading. Samples loaded with drug were used for the *in vitro* release studies. Fifteen replicates were conducted and the weights of loaded biocide per gram of fabric were reported in mean  $\pm$  standard deviation.

### 3.3.7 Release studies

Release of biocides from dried samples was studied by immersing the samples in 10 mL PBS (pH=7.4, 0.1M) which was pre-equilibrated at 32°C. Aliquots (1mL) were taken from samples at different intervals to determine the release concentration. They were then replaced by fresh media. Due to poor absorbance of the biocide in ultraviolet and visible light an indirect spectrophotometric method was used to estimate the biocide concentrations in aliquots.<sup>199</sup> The withdrawing was continued until no increase in absorbance was observed. Experiment was done in quadruplicate and mean  $\pm$  standard error of the mean values were reported.

#### 3.3.7.1 Spectrophotometric method of drug release measurement

Using orange dye II to detect the quaternary ammonium compounds in the media has been reported in literature.<sup>199</sup> Here the positively charged biocides were attached to the negatively charged orange dyes II ( $4 \times 10^{-4}$  M) in 0.1M NaCl; Orange dye-biocide solution was prepared by mixing 4 mL from biocide solution and 2mL orange dye II for 5 minutes. Attached biocides were then extracted from their PBS phase to a chloroform phase by adding 5 mL chloroform to the solution and vortexing for 30 seconds. The two phase solution was allowed to settle for 30 minutes. The bottom phase was then placed for UV-spectroscopy measurement in 485 nm. Similarly blank solution was prepared by extracting a PBS-orange dye II solution in chloroform. The concentrations of biocides were calculated according to a plotted calibration curve containing absorbance of orange dye II at same wavelength.

#### 3.3.7.2 Release behavior analysis

Release in each interval was calculated according to Equation 3:

**Equation 3- Release in each time interval**

$$R_{t_1} \left( \frac{mg}{mL} \right) = \frac{(10C_{t_1} - 9C_{t_0})}{10}$$

In this equation  $R_{t1}$  (mg/mL) is the release in each interval,  $C_{t1}$  (mg/mL) is the current concentration of aliquot and  $C_{t0}$  (mg/mL) is the concentration of aliquot in previous time interval. In cases where R was lower than 0 it was considered as 0 (no release). Cumulative release was calculated by summing up the release in each interval and %loading was calculated by dividing the cumulative release in each time interval per milligram of the loaded biocide. To determine the type of release Fickian formula (Equation 4) was used to analyse the release data until 60% release.<sup>175,176</sup>

**Equation 4- Fickian formula**

$$Mt/M_{\infty} = kt^n$$

According to this equation, ' $M_t/M_{\infty}$ ' is the released drug fraction during time t. 'k' is the kinetic constant including geometric and structural characteristic of delivery system and 'n' is the diffusion exponent suggesting type of the drug transport phenomena. It is worthy of note to point out that this equation is effective only for up to 60% of the release from samples. The value of  $n \leq 0.5$  shows a Fickian diffusion release (non-swell-able matrix) while  $0.5 < n < 1$  indicates non-Fickian release (anomalous) and  $n=1$  is indication of a release which is zero-order.

### **3.3.8 Disk Diffusion test**

Similar to experimental part 1 disk diffusion test was done against both CA-MRSA (40065) and MDR *P. aeruginosa* (73104). The concentrations of CA-MRSA and MDR *P.aeruginosa* were respectively  $2.40E+07$  and  $4.50E+08$  (CFU/mL). Round shape disks with 0.8 cm in diameter were pre-soaked with 1 mL PBS and placed on the Tryptone Soya Agar for 18 hours at 37°C. ZOI diameter was measured in two directions using Vernier callipers, and the average data point  $\pm$  standard deviations were reported.

### **3.4 Statistical analysis**

Results were analysed via Microsoft excel 2010. Mean  $\pm$  standard deviation values were expressed for all experimental data and only mean  $\pm$  standard error of the mean (S.E.M) were presented for biocide release data. Student's t-test was utilized to determine the significance of values and  $p < 0.05$  was considered as statistically significant.

### **3.5 Significance:**

This research is of high industrial importance and the developed technique can be used to improve the adherence profile of existing antibacterial burn wound dressings. In another word, this work will lay a foundation for the construction of non-adherent wound dressings loaded with various suitable topical biocides.

*Chapter 4*  
*Results and Discussions*

## CHAPTER 4. RESULTS AND DISCUSSIONS

### 4.1 Part 1

#### 4.1.1 Peeling force test

In order to choose optimum concentration for acrylamide deposition, different concentrations of polyacrylamide were tested on PET for their swelling ratio and peeling energy. (Figure 1) Peeling energy of samples was calculated in unit area ( $J/m^2$ ). The energy required for the removal of gelatine cast from samples was increased and swelling ratio of hydrogel samples was decreased by decreasing the monomer concentration. Three different pressures were also used during preparation of Acticoat samples.

It was observed that using pressure during hydrogel modification on samples can cause decrease of swelling ratio while maintaining the peeling energy at low level (Figure 2). The peeling energy of Acticoat and Silverlon samples were compared. Untreated Acticoat and Silverlon showed significantly higher peeling energy during removal of gelatine cast than their treated counterparts (Figure 3:  $2070 \pm 453 J/m^2$  and  $669 \pm 68 J/m^2$ , respectively). Deposition of polyacrylamide hydrogel on samples (PAM-10) caused significant decrease in peeling energy of samples to  $158 \pm 119 J/m^2$  for Acticoat and to  $155 \pm 138 J/m^2$  for Silverlon. The obtained peeling energies from polyacrylamide deposited Acticoat or Silverlon are lower than  $300-400 J/m^2$ . Peeling energy lower than  $300-400 J/m^2$  can be considered suitable peeling energy for a drying wound according to Thomas *et al.*<sup>200</sup>

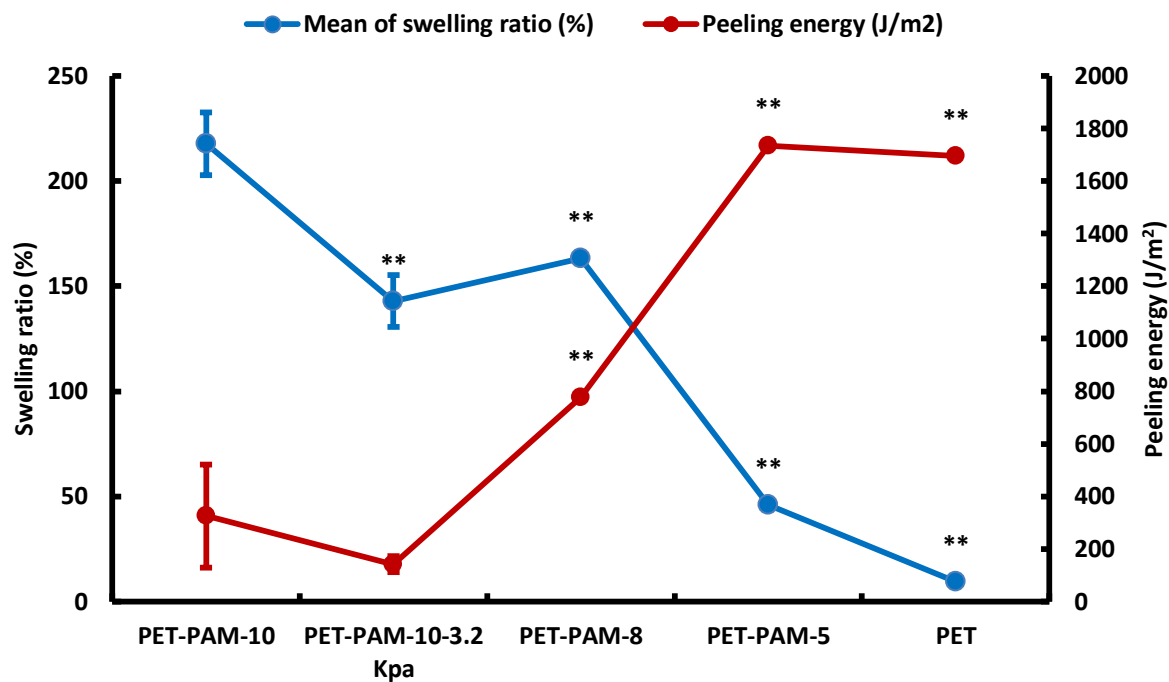


Figure 1- Comparing effect of monomer concentrations on swelling ratio and peeling energy of PET treated samples. Double asterisks indicate swelling ratio and peeling energy values for acrylamide treated samples are significantly different from those of PET-PAM-10. ( $p < 0.05$ )

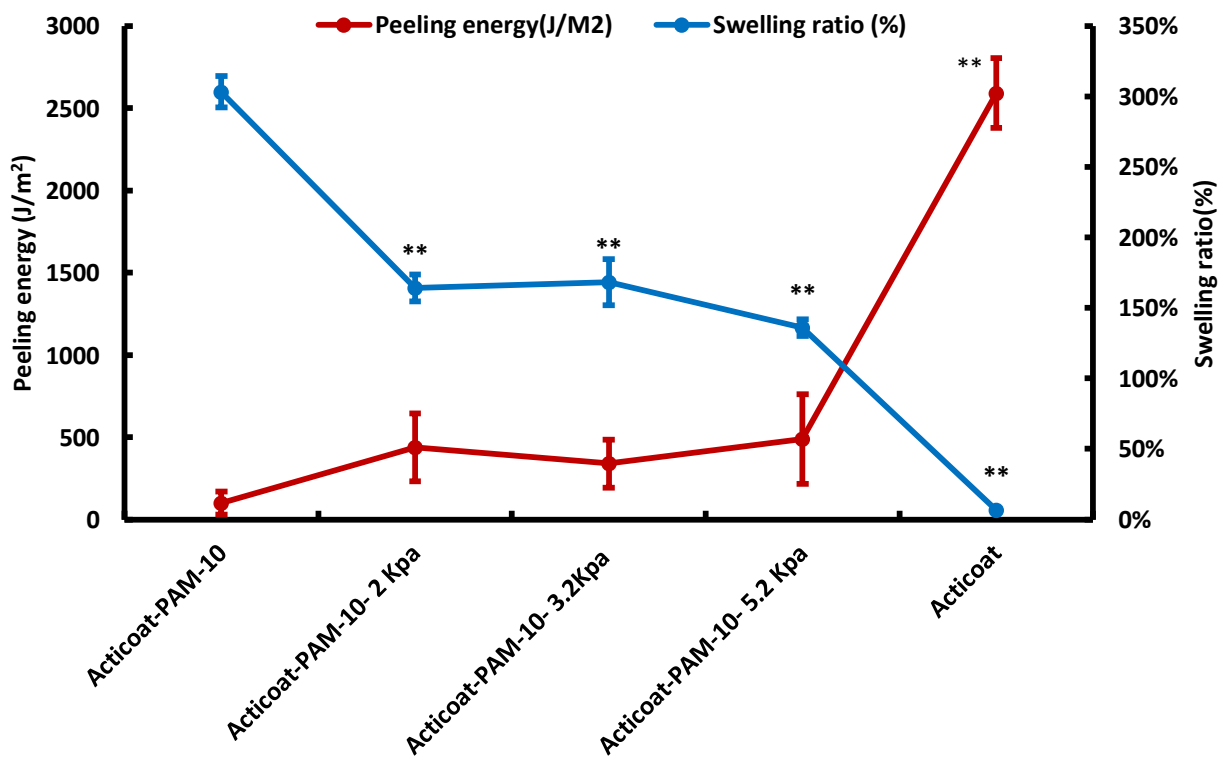


Figure 2- Comparing effect of pressure on swelling ratio and peeling energy of Acticoat treated samples. Double asterisks indicate swelling ratio and peeling energy values for acrylamide treated samples are significantly different from those of Acticoat-PAM-10. ( $p < 0.05$ )

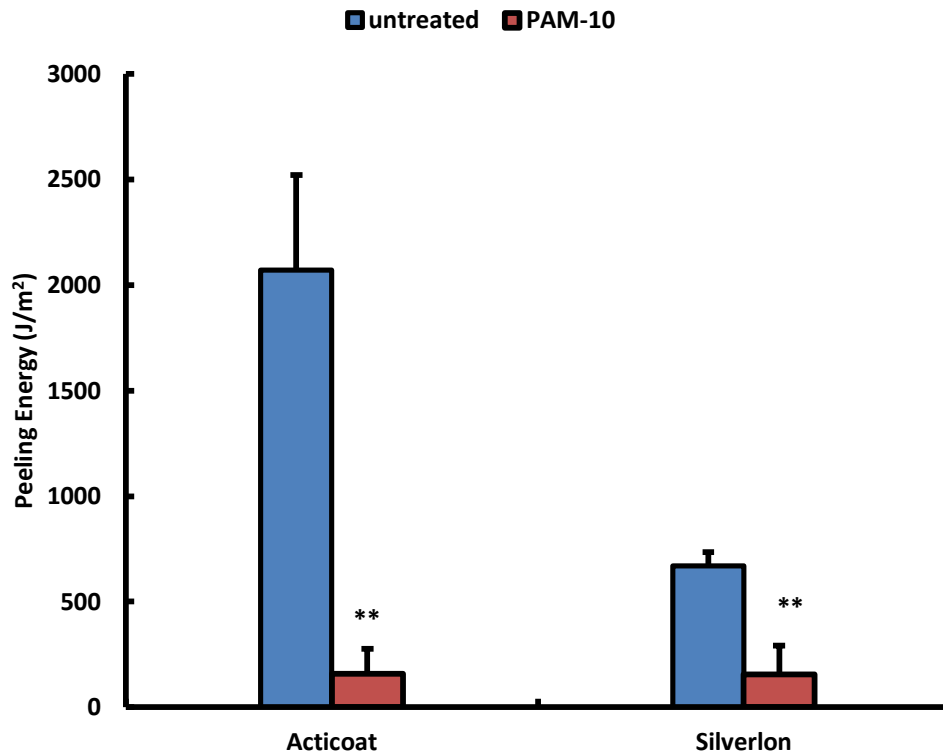


Figure 3- Comparing peeling energy of treated and untreated dressings. Double asterisks significant difference between peeling energy of PAM-10 treated sample and its untreated counterpart ( $p < 0.05$ ).

Exudates –proteinous components- that appear in healing process are the main cause of wound adherence in dressings. These components can penetrate through dressing pores and after being dried can mechanically lock the wound to the dressing. Furthermore they can chemically bond to the dressing through hydrogen bonding.<sup>16</sup> Removal of wound adhered dressing causes damage and pain in newly formed epithelial layer; damage in the newly formed epithelial layer causes delay in the healing.

Our *in vitro* gelatine model was based on a simulation model developed by Andrew *et al.* in which they simulate the wound exudates adherence to the dressing using proteious gelatine. Gelatine can penetrate through the porous of dressing while it is hot and melted and then adhere to dressing’s fine fibres when solidifies upon cooling.<sup>16</sup> Rehman used Andrews’s model to test the adhesion of their bio-degradable film that was designed to prevent adhesion after surgery.<sup>201</sup>

The peeling energy reduction after hydrogel deposition can be explained by pointing out that samples were wetted before gelatine casting. Since the swollen polymeric network of the deposited PAM hydrogel is mostly filled with water gelatine cannot easily penetrate through the network or dressing's pores and therefore the wound dressing will be less adherent to the gelatine slab<sup>133</sup>. Stating that Acticoat Flex 3 is a PET layer covered by silver nanocrystalline, it is possible to conclude that our findings also replicates the results gained by Ning *et al.* in which PAM was used to decrease the adherence of PET to gelatine cast.<sup>162</sup> Likewise, in a submitted thesis by Pu, IPN-PAM modified PET assembled with silver nanoparticles showed 36.25% reduction in gelatine adherence.<sup>161</sup>

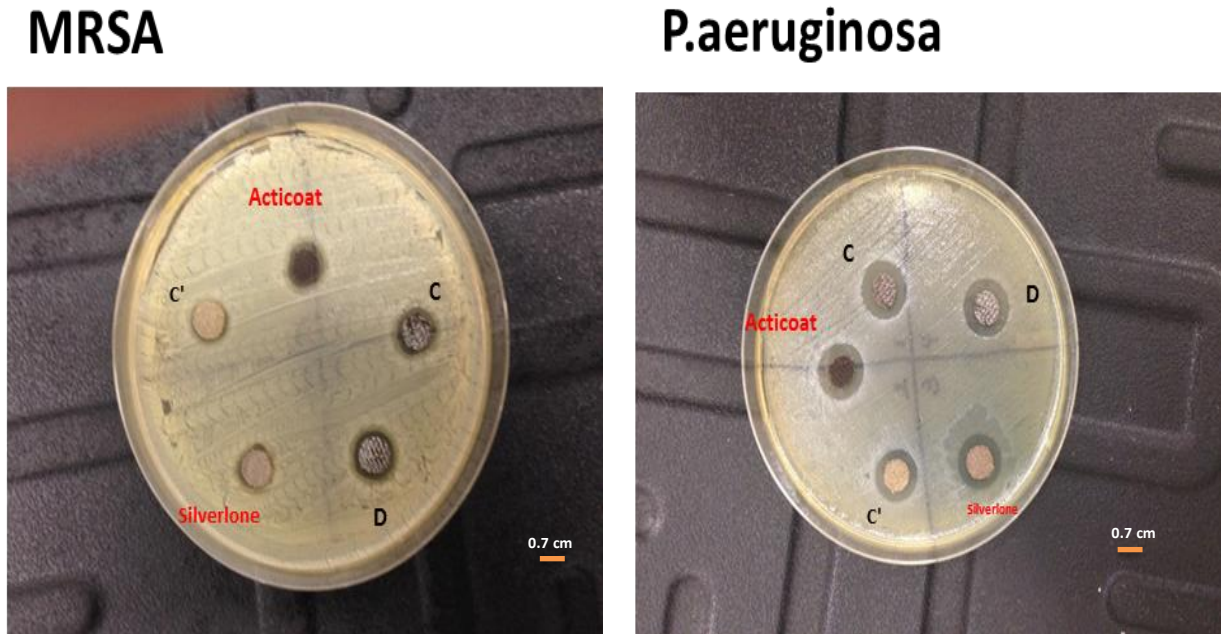
Results from peeling energy showed decreasing the monomer concentration cause increase in the energy required for gelatine removal. Also, adding pressure causes reduction in swelling ratio of samples and did not affected peeling energy. No significant difference ( $p>0.05$ ,  $n=3$ ) was seen in the required peeling energy for gelatine removal when comparing different pressures used for sample preparation and when no pressure was used during sample preparation. Therefore, it was suggested to test other properties of our prepared dressing with and without pressure (PAM-10 and PAM-10-3.2Kpa) either to choose the best condition.

#### **4.1.2 Antimicrobial assays**

Hydrogel network has been used in many studies as a substrate for loading and releasing an antibacterial drug.<sup>202-206</sup> Our concern was to study whether our hydrogel layer affect the antimicrobial ability of the current antimicrobial dressings or not. Thus, the biocidal ability of dressings was tested via an *in vitro* disk diffusion method against bacteria known to typically contaminate burn wounds.

The Zone of Inhibition (ZOI) diameters for dressings are presented in Table 2, Figure 8 and Figure 9 (not autoclaved samples). Untreated and PAM grafted dressings demonstrated no significant difference ( $p>0.05$ ,  $n=6$ ) in the ZOI diameters mean value when tested against

MDR *P.aeruginosa* and CA-MRSA. Nevertheless, as illustrated in Scheme 2, both dressings were less effective against CA-MRSA in comparison to MDR *P. aeruginosa*.



**Scheme 2- Images show inhibition zone of untreated silver dressings, and polyacrylamide treated dressings, against MDR *P. aeruginosa* and CA- MRSA. C and D refers to as Acticoat-PAM-10 and Acticoat-PAM-10-3.2Kpa respectively, while C' refers to Silverlon-PAM-10**

As mentioned, investigating the results in more detail showed both dressings effect better against the gram negative MDR *P.aeruginosa*, in comparison with gram positive CA-MRSA. Boonkaew *et al.* have tested hydrogels containing silver nanoparticles antibacterial ability against *P. aeruginosa* and MRSA and similarly reported less susceptibility of gram positive MRSA to silver ions compare to gram negative *P. aeruginosa*. They explained their results considering two points: first, it is possible that the cationic silver ions entrapped in the negatively-charged peptidoglycan's cell-wall of gram-positive MRSA and therefore caused them to be less effective. Second, because the gram-positive bacteria have thicker cell wall compare to gram-negative bacteria, the former bacteria are more resistant to silver compare to the latter. <sup>207</sup>

As stated, our results showed no compromise in the antibacterial ability of the wound dressings. According to Xiu *et al.*, toxicity of silver nanoparticles to bacteria are due to released  $\text{Ag}^+$  generated from oxidation of  $\text{Ag}(0)$  and the silver nanoparticles do not demonstrate any direct particle-specific bacterial toxicity.<sup>51</sup> The nano-crystalline silvers in Acticoat dressings are the sources of the antimicrobial ionic silver.<sup>52</sup> Thus, it is important to study the effect of treatment on silver release from samples to better understand the effect of PAM deposition on antimicrobial ability of the samples. Following in section 4.1.3 the concentration of released  $\text{Ag}^+$  from Acticoat samples in predetermined time intervals are investigated.

**Table 2- Zone of inhibition diameters (mm) of dressings tested against MDR *P.aeruginosa* and CA-MRSA. The resulting concentrations were respectively 1.00E+08 and 1.29E+08 (CFU/mL) for MDR *P.aeruginosa* and CA-MRSA.**

<i>Microorganisms</i>	<i>Acticoat</i>	<i>Acticoat-PAM-10</i>	<i>Acticoat-PAM-10-3.2Kpa</i>	<i>Silverlon</i>	<i>Silverlon-PAM-10</i>	<i>Silverlon-PAM-10-3.2Kpa</i>
MDR <i>P.aeruginosa</i>	10.8±0.89	11.23±0.62	11.47±0.23	10.33±0.73	9.72±0.42	10.27±0.82
CA-MRSA	8.5± 1.11	8.63±0.70	8.38±0.18	8.27± 0.83	7.98±0.34	7.83±0.50

In addition, to confidently state that the contact of the un-coated-edges of the dressing (Respectively 4% and 11% of total surface area of Acticoat and Silverlon) with agar surface is not the certain reason allowing direct diffusion of silver into the agar and create the zones of inhibition that were seen; in another coating experiment edges of samples were covered with polyacrylamide and test was repeated with the edge coated samples. As evidence Scheme 3 is presented showing the coating around the samples. Hydrogel thickness was estimated to be 337.83  $\mu\text{m}$  for Acticoat-PAM-10 and 515.67  $\mu\text{m}$  for Silverlon-PAM-10.



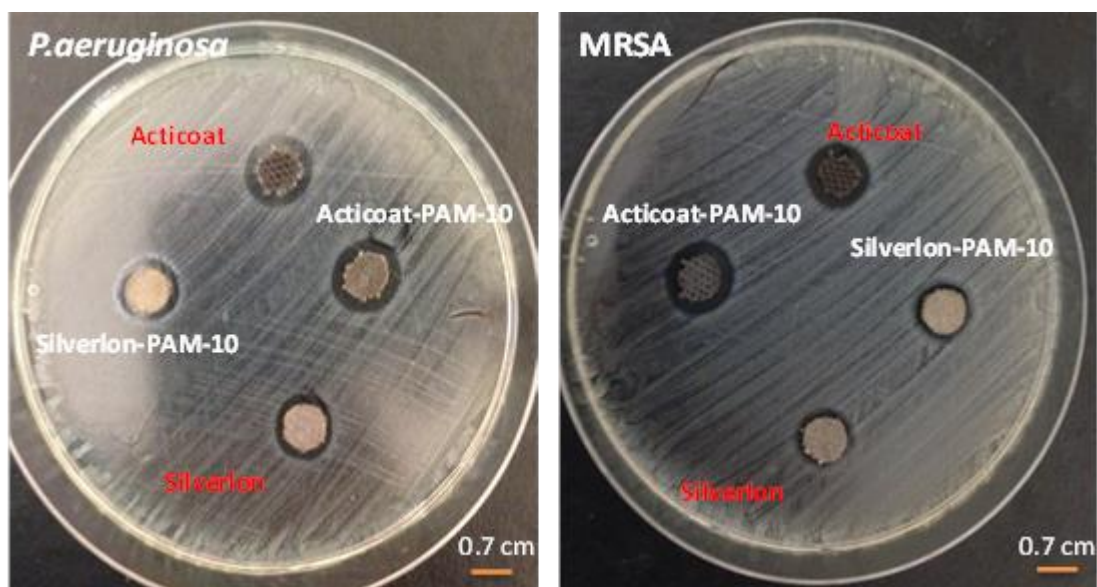
**Scheme 3- Observation of Acticoat dressing before and after coating their edges under the microscope. Left pictures are Acticoat-PAM-10 and right pictures are Acticoat sample.**

Following sample preparation another disk diffusion assay was conducted with the newly prepared samples. The MDR *P.aeruginosa* concentration was  $7.8E+07$  CFU/mL and CA-MRSA concentration was  $7.3E+07$  CFU/mL.

**Table 3- Zone of inhibition diameters (mm) of dressings tested against MDR *P.aeruginosa* and CA- MRSA with the edge coated samples**

<i>Microorganisms</i>	<i>Acticoat</i>	<i>Acticoat-PAM-10</i>	<i>Silverlon</i>	<i>Silverlon-PAM-10</i>
MDR <i>P.aeruginosa</i>	12.2±0.49	12.55±0.40	10.65±0.69	10.97±0.40
CA-MRSA	10.75± 0.80	11.62±0.77	9.45± 0.61	9.63±0.49

As presented in Table 3 and Scheme 4, again, zone of inhibition (ZOI) data of both dressings are not significantly different before and after the deposition of PAM layer (t-test,  $P>0.05$ , n=6).



Scheme 4- Images showing comparison between inhibition zones of edge coated samples and untreated samples.

As stated before to rule out the possibility that direct release of ionized silver from the un-grafted edges of the dressing samples contributes significantly to the observed zone of inhibition in Table 2 and Scheme 2, we also grafted PAM hydrogel onto pre-cut dressing samples (1×1 cm<sup>2</sup>, for suspension antibacterial test). Following this sample preparation, a suspension antibacterial test was conducted. The results from the suspension test are presented in

Table 4 and Table 5. We can see that the PAM layer did not significantly ( $P>0.05$ ,  $n=3$ ) compromised the antimicrobial ability of the silver dressings.

Table 4- Antimicrobial efficiency of dressings tested against CA-MRSA.

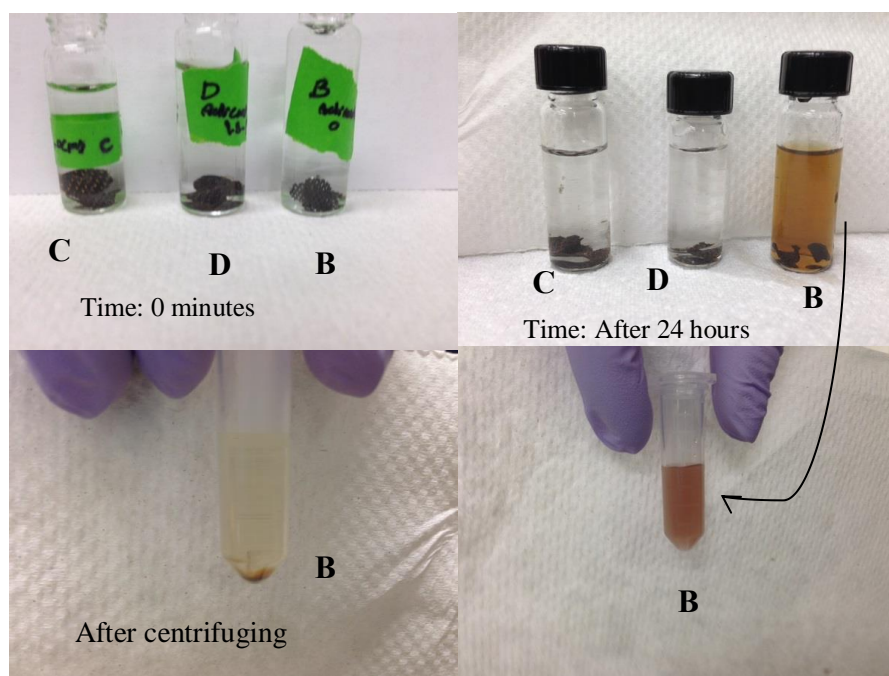
Sample name	30 min		2h		24h	
	%	Log10	%	Log10	%	Log10
Acticoat	14.37±8.81	0.07±0.05	100.00±0.00	6.25±0.00	100.00±0.00	6.21±0.00
Acticoat-PAM-10	6.16±9.24	0.03±0.04	100.00±0.00	6.25±0.00	100.00±0.01	6.21±0.00
Silverlon	0	0	35.56±16.80	0.2±0.11	99.99±0.01	5.49±1.26
Silverlon-PAM-10	0	0	0	0	99.99±0.01	5.49±1.26

**Table 5- Antimicrobial efficiency of dressings tested against MDR *P.aeruginosa*.**

Sample name	1h		2h		24h	
	%	Log10	%	Log10	%	Log10
Acticoat	100.00±0.00	6.36±0.00	100.00±0.00	6.48±0.00	//	
Acticoat-PAM-10	100.00±0.00	6.36±0.00	100.00±0.00	6.48±0.00	//	
Silverlon	62.40±20.33	0.48±0.27	99.69±0.16	2.57±0.27	99.97±0.01	3.71±0.13
Silverlon-PAM-10	57.05±24.58	0.41±0.26	99.88±0.05	2.93±0.18	99.93±0.05	3.19±0.27

### 4.1.3 Silver release test

To better understand the effect of PAM deposition on the release of ionic silver from Acticoat dressings the concentration of  $Ag^+$  released from samples was measured via ICP-OES. After withdrawing the aliquots in proper time intervals (30 minutes, 2 hours and 2 days) the insoluble silver particles (Scheme 5) were removed from the aliquots by centrifuging at 14000 rpm for 10 minutes.



**Scheme 5- Released silver particles from treated and untreated Acticoat samples after 24 hours in DI water. Samples from left to right are: B: Acticoat, C: Acticoat-PAM-10, D: Acticoat-PAM-10-3.2Kpa.**

The cumulative concentrations of released silver ions in each time interval are presented in Figure 4. A significant difference ( $P < 0.05$ ,  $n=3$ ) was seen in the concentration of cationic silver released from Acticoat-PAM-10 ( $5.12 \pm 3.73$  ppm) and Acticoat-PAM-10-3.2Kpa ( $5.57 \pm 3.84$  ppm) with the untreated Acticoat ( $15.24 \pm 0.17$  ppm) 30 minutes after immersing the samples in DI water. Nevertheless, this difference have grown smaller after 2 hours (Acticoat-PAM-10:  $16.63 \pm 1.49$  ppm, Acticoat-PAM-10-3.2Kpa:  $12.37 \pm 5.99$  ppm and untreated Acticoat:  $20.94 \pm 1.29$  ppm).

After 48 hours no significant difference ( $P > 0.05$ ,  $n=3$ ) was seen on the concentrations of released silver from all three conditions (Acticoat-PAM-10:  $86.61 \pm 3.65$  ppm, Acticoat-PAM-10-3.2Kpa:  $71.83 \pm 18.37$  ppm and untreated Acticoat:  $83.38 \pm 3.39$  ppm). The PAM hydrogel layer delays  $Ag^+$  release in the first 2 hours but does not impact significantly on the overall amount of  $Ag^+$  in 48 hours. The amount of released silver from untreated Acticoat after 30 minutes, 2 hours and 48 hours are respectively comparable to those obtained by Rigo *et al.*<sup>50</sup>

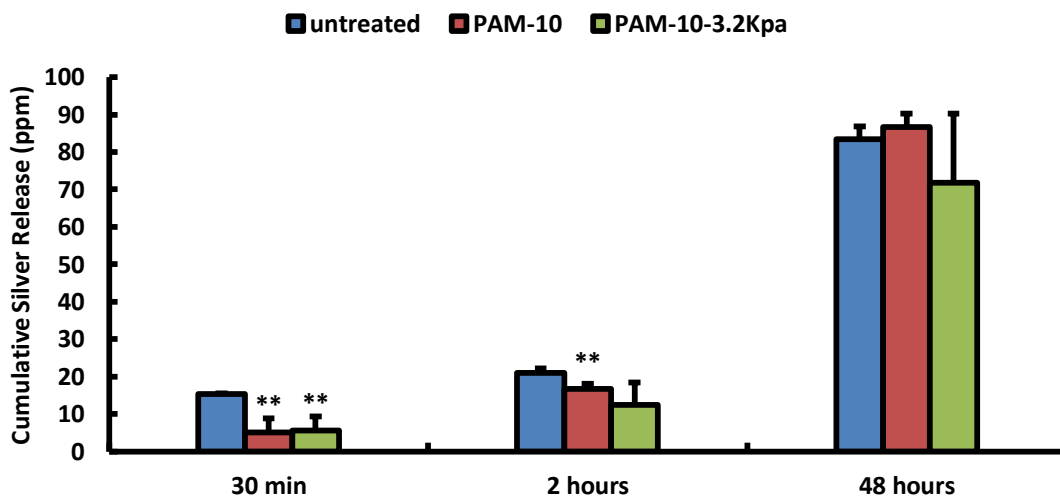


Figure 4- Comparing cumulative silver release concentration of Acticoat Flex 3 samples after 30 minutes, 2 hours and 48 hours. Double asterisks indicates significant difference between PAM treated and untreated values at each time interval ( $p < 0.05$ ,  $n=3$ ).

#### 4.1.4 Cytotoxicity test

As burn wounds are Cl<sup>-</sup> rich environments; we considered the cytotoxicity test both in DI water and saline medium. Also as previously explained because both keratinocytes and fibroblasts cells have viable role in healing process, the cytotoxicity of the dressings on both of these cells were tested.

Obtained data from cytotoxicity test in fibroblast cells demonstrated 41±12% cellular viability in water and 48±5% in saline for untreated Acticoat dressings. Similarly viable cells after testing the untreated Silverlon were 55±8% in saline and 67±4% in water. The PAM grafted samples showed higher ( $p < 0.05$ ,  $n = 12$ ) cell viability (Figure 5) compared to the untreated ones both in saline and water tests. Acticoat-PAM-10 cell viability in water and saline were respectively 60±11% and 60±3%. A slight increase was also seen in the viability of fibroblast cells for the PAM grafted Silverlon dressings compared to the untreated Silverlon (correspondingly 73±3% and 61±4% in water and saline). Samples prepared with pressure PAM-10-3.2Kpa also showed higher or similar cell viability (Acticoat-PAM-10-3.2Kpa cell viability in water and saline were respectively 51%±3% and 49%±10% also their Silverlon counterparts showed 74%±6% and 71%±9% fibroblast cells viability in water and saline correspondingly).

Results of cytotoxicity testing on keratinocyte cells (Figure 6) exhibited higher cell viability percentage compare to fibroblast cells. In case of untreated Acticoat samples 81%±5% cell viability was observed in saline and 76%±3% in water. Similarly 80%±7% cell viability was observed in saline and 77%±9% in water for untreated Silverlon samples. Treatment of the dressings with PAM layer did not increased their cytotoxicity. In case of Acticoat dressings the treatment caused significant ( $p < 0.05$ ,  $n = 12$ ) increase in the keratinocyte cells viability. (Acticoat-PAM-10: 97%±4% in water and 98%±9% in saline also

Acticoat-PAM-10-3.2Kpa showed 99%±4% and 88%±10% cell viability in water and saline correspondingly)

Also as mentioned burn wound are  $\text{Cl}^-$  reach environment thus, the effect of the dressings on cell viability was studied both in saline and DI water. Presence of released  $\text{Ag}^+$  in a  $\text{Cl}^-$  reach environment will lead to the formation of  $\text{AgCl}$  salt which are insoluble and tend to deposit. This process will lead to deactivation of the dressing by preventing  $\text{Ag}^+$  development and transfer.<sup>50</sup> Furthermore, cells are able to pick insoluble particles by their endocytosis mechanism this process can lead to cell's toxicity.<sup>50</sup> That could be the reason that in most cases no reduction was seen in the cytotoxicity of the dressing in saline compare to their counterpart in water and even in some cases a significant increase was seen in cytotoxicity of the dressing when testing the dressing in saline compare to its counterpart in water.<sup>49</sup>

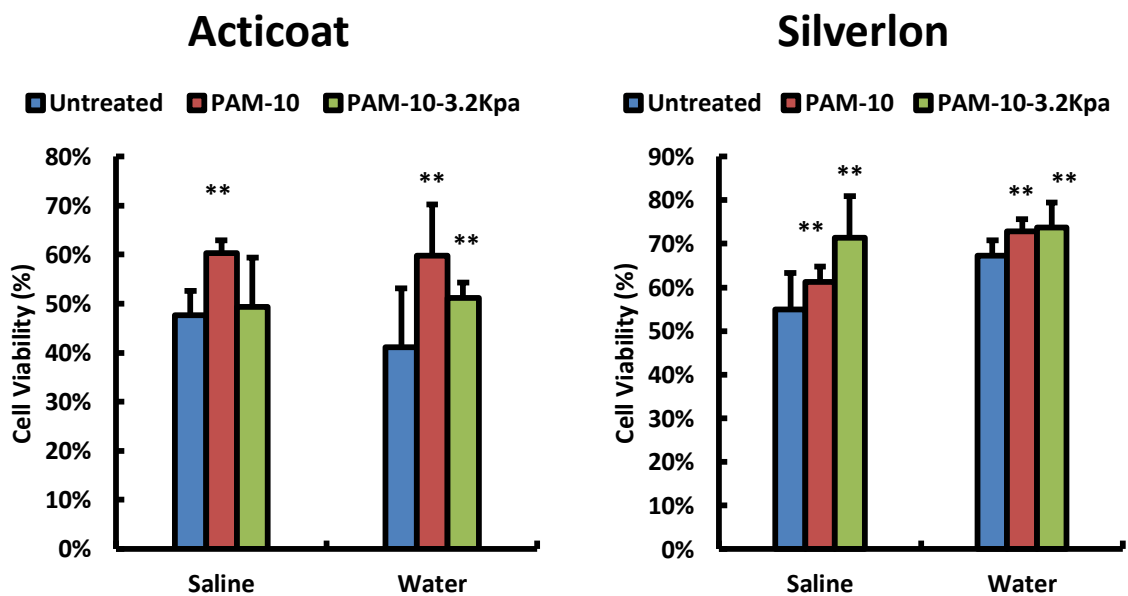


Figure 5- Evaluation of relative Fibroblast cell viability after 24 hours dressing treatment. The exhibited results are percentage of viable cells of untreated Acticoat and Silverlon dressings or polyacrylamide grafted dressings to the control group both in the culturing media containing saline or water. Double asterisks indicates significant difference between PAM treated and untreated values in each culturing media ( $p < 0.05$ ,  $n = 12$ ).

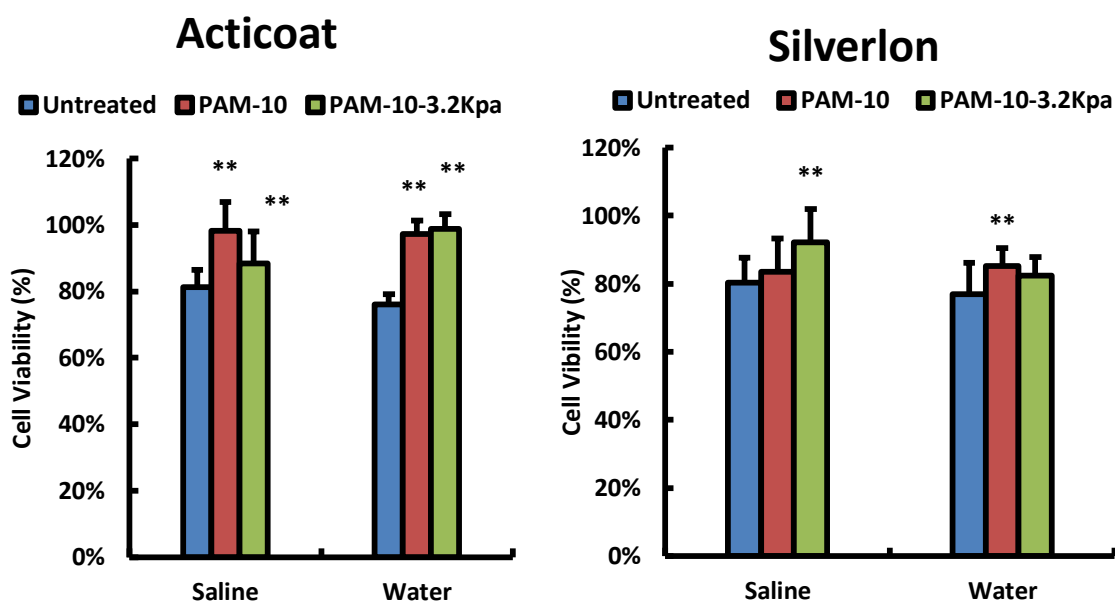


Figure 6- Evaluation of relative keratinocyte cell viability after 24 hours dressing treatment. The exhibited results are percentage of viable cells of untreated Acticoat and Silverlon dressings or polyacrylamide grafted dressings to the control group both in the culturing media containing saline or water. Double asterisks indicates significant difference between PAM treated and untreated values in each culturing media ( $p < 0.05$ ,  $n = 12$ ).

Our results showed that hydrogel deposition (PAM-10 and PAM-10-3.2Kpa) on Acticoat and Silverlon can increase the percentage of viable cells. The reduction in cytotoxicity with persistence of antibacterial activity was an unexpected finding. There are a number of possible explanations for how the addition of the PAM layer may affect this.

There is some evidence that suggests toxicity of silver nanoparticles to bacteria is due to released  $Ag^+$  generated from oxidation of  $Ag(0)$  and the silver nanoparticles themselves do not exert any direct particle-specific bacterial toxicity<sup>51</sup>. Conversely eukaryotic cells are more prone to damage from silver nanoparticles as they readily take up silver particles via endocytosis and other mechanisms. In one example, Bressan *et al.* present how silver nanoparticles can in addition cause cytotoxicity in human dermal fibroblasts through disturbing the respiratory system by accumulating outside the mitochondria<sup>208</sup>.

It maybe that the PAM layer acts as a physical barrier, whereby it reduces the release of silver nanoparticles (Scheme 5 confirm this statement) but does not interfere as much with the release of  $Ag^+$  (Figure 4 confirms this statement by showing no significant difference in

the cumulative  $\text{Ag}^+$  release after 24 hours). Furthermore as previously reported MIC value of cationic silver were reported to be 2-4 ppm<sup>181,187</sup> and as shown in Figure 4 even in the initial hours of silver release the concentration of released  $\text{Ag}^+$  is still higher than this value. Thus no significant change was seen in the antimicrobial ability of the samples.

Also it is worthy of note to mention that during the procedure of grafting cross-linked PAM layer on Acticoat samples, release of certain silver nanoparticles from these samples was observed (solution turned into a brownish colour), thus less silver particle are available to be released from treated samples during cytotoxicity testing. Considering the higher release of silver particles from the treated samples compare to untreated it is possible to explain lower toxicity of the PAM treated samples.

Previous reported techniques have used a layer by layer design, similar to our own, to immobilize the silver nanoparticles maintaining antibacterial effect with no cytotoxicity<sup>209</sup>. As well it has been shown that the toxicity threshold is much broader for human cells than for bacteria<sup>49</sup>.

#### **4.1.5 Effect of autoclaving on peeling energy and antibacterial ability of samples**

To prevent the risk of pathogen invasion it is essential to sterilize and disinfect the material used in clinic.<sup>210</sup> Since the cytotoxicity test was done after autoclaving, studying the effect of autoclaving on the adherence and antibacterial properties of samples was suggested.

The energy required to remove the gelatine cast was measured after Acticoat samples were sterilized by autoclaving at 121°C. Each condition was done in triplicate. During the peeling test of autoclaved untreated samples two out of three of the gelatine casts were broken due to high lockage of gelatine to the porous structure of the untreated Acticoat samples. The third pristine sample showed higher peeling energy (4917 J/m<sup>2</sup>) compare to its not-autoclaved counterpart (2069±452 J/m<sup>2</sup>). Also comparing autoclaved PAM grafted Acticoat samples with their not-autoclaved counterparts (Figure 7) showed steam sterilization causes increase

in the adherence of samples to the gelatine cast (autoclaved Acticoat-PAM-10:1347±385 and not-autoclaved Acticoat-PAM-10:157±119 J/m<sup>2</sup>).

The effect of autoclave sterilization method on the antibacterial ability of PAM treated samples was also studied by comparing the diameter of inhibition zone created by autoclaved samples and their not-autoclaved counterparts.

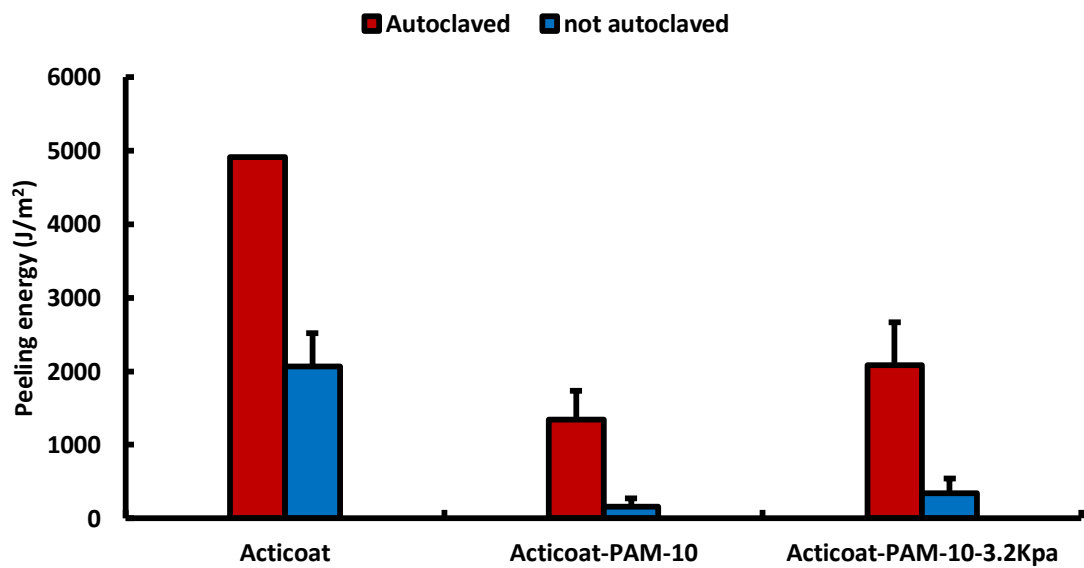


Figure 7- Comparing peeling energy of autoclaved samples with not-autoclaved samples

As presented in Figure 8 and Figure 9 the autoclaved PAM-treated samples showed significantly smaller ZOI ( $p < 0.05$ ,  $n = 6$ ) against both CA-MRSA and MDR *P.aeruginosa* compare to the not-autoclaved PAM-treated samples. It is noteworthy to mention that autoclaving did not affect the ZOI of untreated samples.

Steam sterilization is a method which has been traditionally used for sterilizing medically used materials.<sup>211</sup> According to the results peeling energy and disk diffusion assay, steam sterilization causes increase in adherence of treated samples to the simulated wound exudates and reduction in the antimicrobial performance of the treated samples. This could be due to the hydrolysis of acrylamide hydrogel in presence of high temperature and steam during autoclaving procedure. In other words, the hydrogel network will become degraded because

of brakeage in C-N bond in polyacrylamide and the gelatine can penetrate easier through the network. Therefor the peeling energy required for removal of gelatine increases.

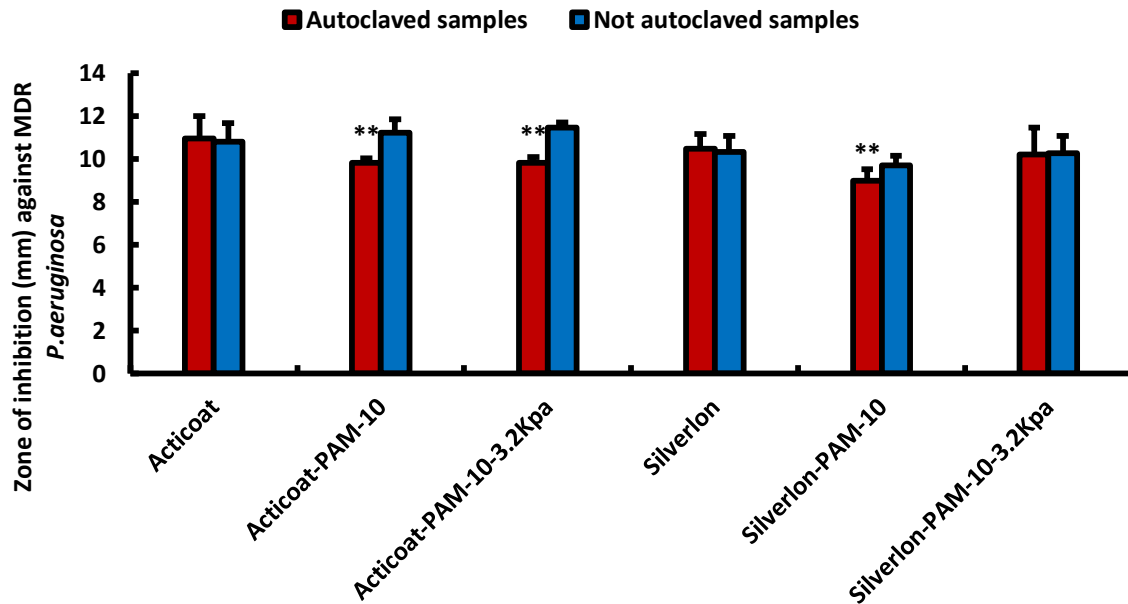


Figure 8- Comparing ZOI value of autoclaved and not-autoclaved samples against MDR *P.aeruginosa*, (1.00E+08 CFU/mL). Double asterisks indicates significant different (p<0.05, n=6) between ZOI value of autoclaved samples and their not-autoclaved counterparts.

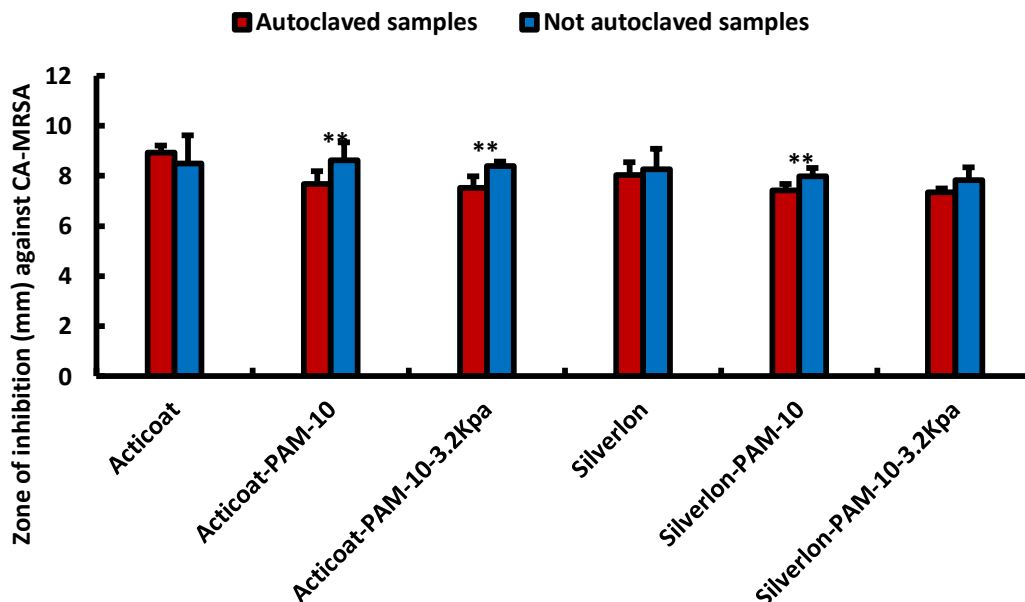


Figure 9- Comparing ZOI value of sterilized and non-sterilized samples against CA-MRSA (1.29E+08 CFU/mL). Double asterisks indicates significant difference (p<0.05, n=6) between ZOI value of autoclaved samples and their not-autoclaved counterparts.

Similarly due to degradation of hydrogel diffusion of silver nanoparticles can be compromised and the biocidal ability of the treated samples will be reduced. As a confirmation, Romero *et al.* observed reduction in tensile strength of poly (vinyl alcohol) hydrogels after 5 minutes autoclaving at 121°C in different timings.<sup>212</sup>

Another question pops when looking at Figure 7 and comparing the peeling energy of autoclaved and not autoclaved untreated Acticoat. This question is: Why the autoclaving procedure affected the peeling energy of untreated Acticoat sample and caused increase of their peeling energy?

To answer this question, it is worthy of note to mention that during the cytotoxicity experiment higher hydrophilicity was observed in the autoclaved untreated Acticoat samples compare to the not-autoclaved untreated Acticoat samples. The hydrophilicity helps better penetration of the gelatine through the dressing and increases the peeling energy. As a confirmation a swelling ratio test was conducted to compare the hydrophilicity of autoclaved and not autoclaved untreated Acticoat. Autoclaved Acticoat showed significantly higher ( $p < 0.05$ ,  $n=3$ ) swelling ratio ( $112 \pm 8\%$ ) compare to the not-autoclaved Acticoat ( $9 \pm 3\%$ ).

According to smith & nephew Acticoat Flex 3 is made of “single layer of knitted polyester”<sup>213</sup>. FTIR spectra from both untreated Acticoat and polyester were taken to confirm that Acticoat has similar structure to the PET. As shown in Figure 10 Peaks at 1713 (C=O), 1336 (CH<sub>2</sub>), 965 (Trans configuration of ethylene glycol unit), 897 (Gauche configuration of –OCH<sub>2</sub>CH<sub>2</sub>- group), 794 (Benzene ring), in Acticoat overlap with those of PET and matches with the peaks reported in polyester via Tracey *et al.*<sup>214</sup>

The swelling ratio test was also conducted on an autoclaved and not-autoclaved pristine PET sample to investigate the effect of autoclaving on the PET structure. The swelling ratio for the not-autoclaved-PET samples were  $13 \pm 3\%$  and the autoclaved samples were  $18 \pm 5\%$ .

Obtained data showed that the Autoclaving procedure does not significantly ( $P > 0.05$ ,  $n=3$ ) affect the swelling ratio of untreated-polyester (PET)-samples.

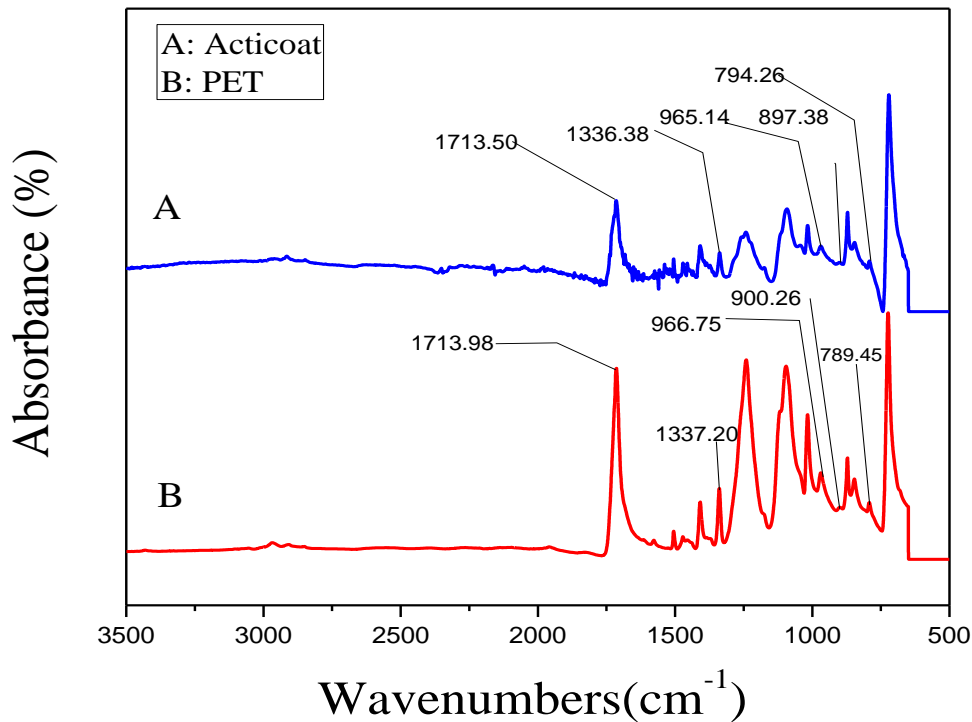


Figure 10- ATR-FTIR spectra of untreated Acticoat and PET.

A possible explanation for the increase in the adherence of samples to the gelatin cast could be the degradation (exposing more hydroxyl group) of Acticoat after plasma deposition of silver-nano-particles (AgNPs). Other possible explanation could be the removal of certain amounts of hydrophobic AgNPs due to autoclaving which lead to more hydrophilicity of the autoclaved-Acticoat samples and their higher adherence to the gelatine model.

#### 4.1.6 Effect of ethylene oxide sterilization on peeling energy antibacterial ability of samples

Ethylene oxide sterilization was suggested as another method to be use for sterilizing samples. Ethylene oxide sterilization is a compatible method for variety of medical devices

and materials, especially for heat or moisture sensitive materials compare to other sterilization methods such as  $\gamma$  or steam which can cause physical damage to the materials and reduce their performance. Polymer degradation and cleavage of chemical bonds are examples.<sup>215</sup> As shown in Figure 11 ethylene oxide sterilization did not significantly ( $P>0.05$ ,  $n=3$ ) affected the required peeling energy for removal of gelatin cast.

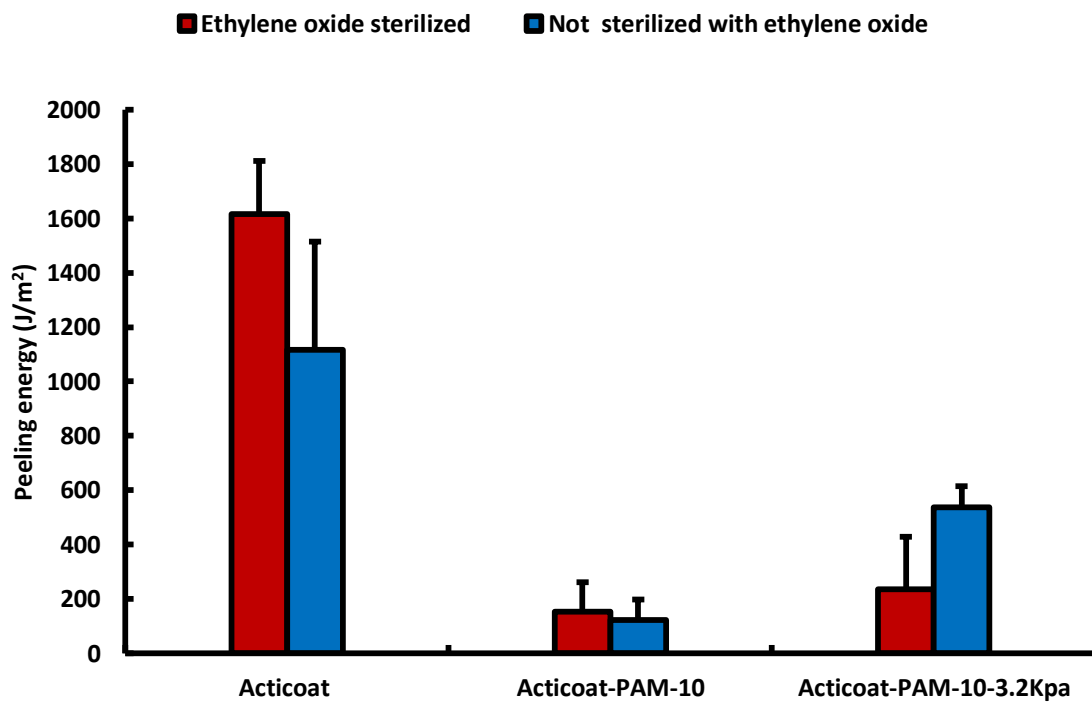


Figure 11- Comparing peeling energy of ethylene oxide sterilized samples with not sterilized samples.

To determine whether ethylene oxide sterilization method affects the biocidal capability of samples or not, sterilized and not sterilized samples were also challenged against both CA-MRSA ( $5.5E+07$  CFU/mL) and MDR *P. aeruginosa* ( $8.00E+08$  CFU/mL) isolates. As shown in Figure 12 and Figure 13, the ethylene oxide sterilization did not significantly ( $p>0.05$ ,  $n=6$ ) affected the ZOI diameter of samples against both bacteria strains. These results shows that it is possible to consider the ethylene oxide sterilization method as a substituted method for autoclaving.

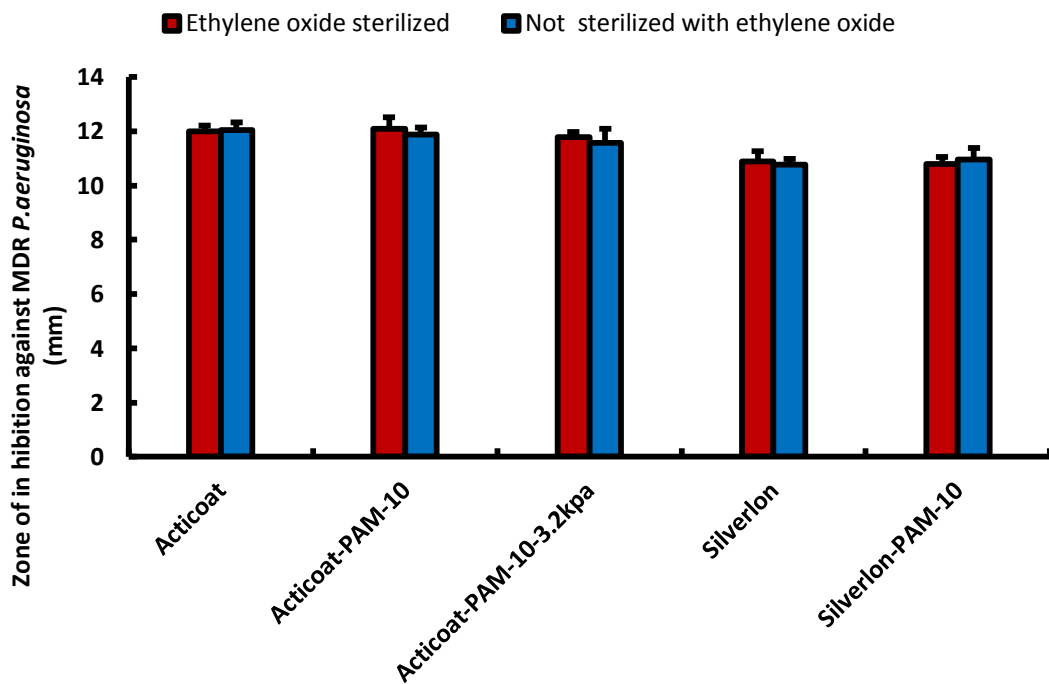


Figure 12- Comparing ZOI value of ethylene oxide- sterilized and not-sterilized samples against MDR *P.aeruginosa*, (8.00E+08 CFU/mL).

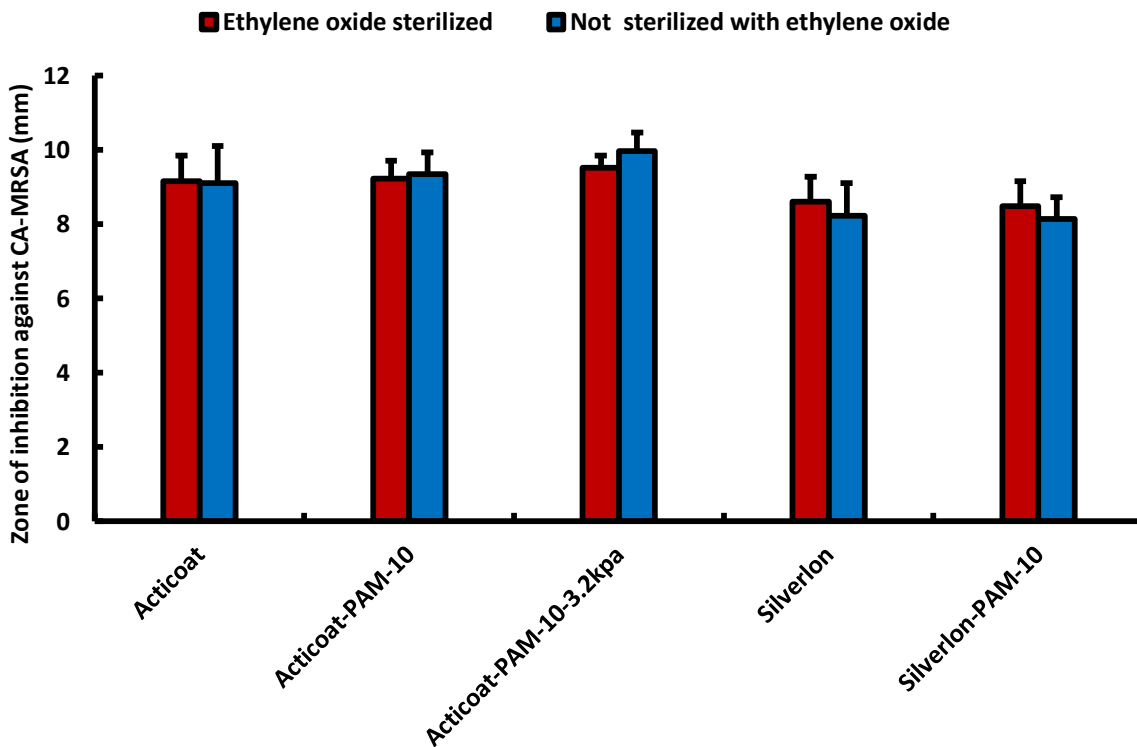


Figure 13- Comparing ZOI value of ethylene oxide- sterilized and not-sterilized samples against CA- MRSA, (5.5E+07 CFU/mL).

## 4.2 Part 2

### 4.2.1 Deposition of acrylamide-*co*-acrylic acid hydrogel on PET

Among different techniques to prepare hydrogel (chemical freeze-thawing, irradiation) irradiation is a suitable method since it is easy to control and can be done in one step. Also there is no need for initiators which are mostly harmful to human body.<sup>216</sup> In this study, different ratios of acrylic acid and acrylamide were deposited on the PET dressing via UV-irradiation. The aim was to introduce a negative charge in the hydrogel structure. Samples were then characterized for their swelling ratio, carboxylate group concentration and adherence.

Weight of loaded hydrogel on samples was calculated by deducting the weight of dressings before and after loading hydrogel and weight increment was calculated by dividing this number per weight of samples before hydrogel loading. As seen in Figure 14 and Table 6 PET-PAM-PAA-9-1 and PET-PAM-PAA-7-3 samples showed significantly ( $P < 0.05$ ,  $n = 10$ ) higher weight increment compare to the PET-PAM-10 samples.

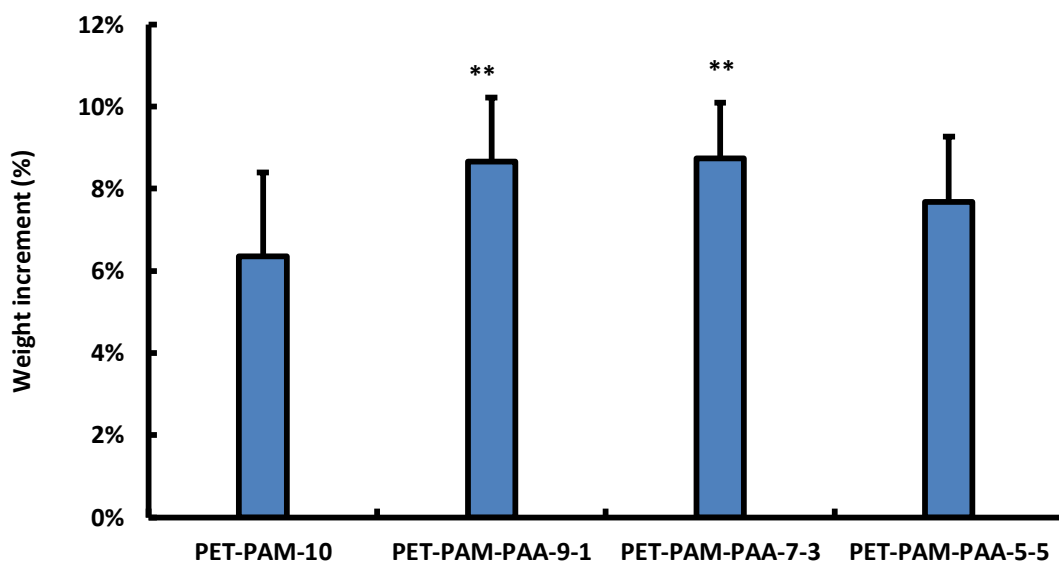


Figure 14- Weight increment of samples after hydrogel deposition. Double asterisks indicate significant difference ( $p < 0.05$ ,  $n = 10$ ) between the weight increment value of PET-PAM-10 and other values.

Swelling ratio test was used to investigate whether or not substituting part of PAM structure via acrylic acid can affect the swelling capacity of samples. The test was conducted in PBS (pH=7.4, 0.1M). It is worthy of note to point out that the pKa of acrylic acid is 4.2; which means acrylic acid can dissociate in PBS (pH=7.4) and more than 50% of its carboxyl groups will be ionized (having negative charge).<sup>217,218</sup>

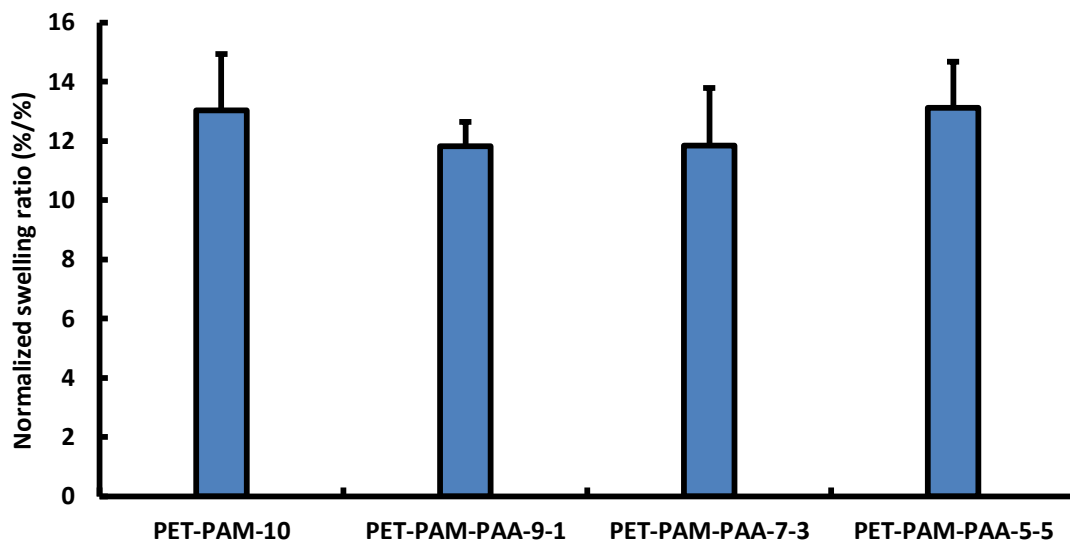


Figure 15- Comparing normalized swelling ratio in samples. No significant difference ( $p < 0.05$ ,  $n = 8$ ) was seen between the normalized swelling ratio value of PET-PAM-10 and other values.

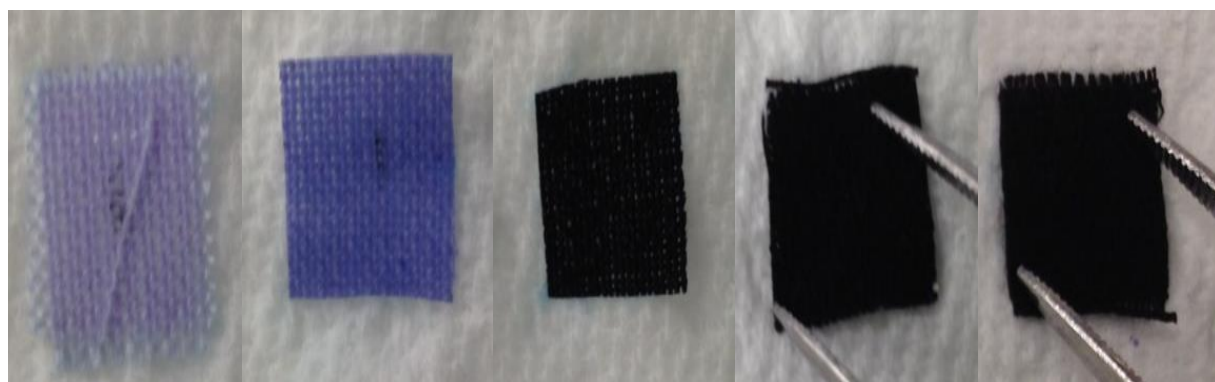
To remove effect of hydrogel weight and better study the effect of acrylic acid existence on the swelling ratio of samples in PBS, the swelling ratio of samples in PBS were divided per weight increment of samples. The normalized swelling ratio is presented in Table 6 and Figure 15. It was expected that samples containing acrylic acid would show higher swelling compare to PET-PAM-10 samples due to the repulsion between the negative charges in their structure. However, comparing the normalized swelling ratio of PET-PAM-10 samples and PET-PAM-PAA samples showed no significant ( $p > 0.05$ ,  $n = 8$ ) difference. This might be due to the suppression of double-electric-layer by neutral salt in PBS (0.1 M).

**Table 6- Weight increment of samples and normalized swelling ratio (%)**

<i>Samples</i>	<i>Weight increment after hydrogel loading hydrogel (%)</i>	<i>normalizing swelling ratio</i>
PET-PAM-10	6±2%	13.03±1.90
PET-PAM-PAA-9-1	9±2%	11.82±0.81
PET-PAM-PAA-7-3	9±1%	11.83±1.94
PET-PAM-PAA-5-5	8±2%	13.11±1.56

#### **4.2.1.1 Determination of carboxylic acid content on samples**

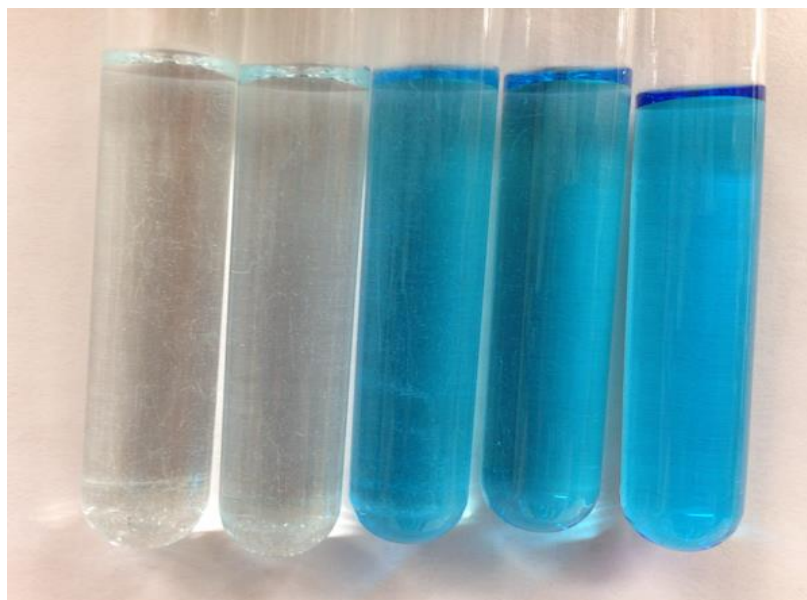
Colorimetric titration based on an absorption test via Toluidine Blue O (TBO) from Ma *et al.*<sup>198</sup> was used to quantify the carboxylic acid content of the samples. TBO as a blue dye that bears positive charge is able to form complex with anionic carboxylate group in the acrylic acid in a 1:1 ratio due to the attraction between the two opposite charge.<sup>165,198</sup> The absorption of this dye by hydrogel treated dressings was used to measure the concentration of carboxyl group in the structure of hydrogel.



**Scheme 6- Sample's color after immersion in TBO solution for 30 minutes. Samples from left to right are: PET, PET-PAM-10, PET-PAM-PAA-9-1, PET-PAM-PAA-7-3 and PET-PAM-PAA-5-5.**

Scheme 6 shows the dye absorbed by samples after 30 minutes immersion in TBO solution (pH=10). Also the released TBO from samples after immersion in 50% acetic acid

are presented in Scheme 7. Released TBO from samples containing acrylic acid are 10 times diluted before UV/vis measurement.



**Scheme 7- The released TBO from samples after placing them in 50% acetic acid for 1 hours (samples containing acrylic acid were 10× diluted for analyzing in UV-Spectroscopy). Samples from left to right are: PET, PET-PAM-10, PET-PAM-PAA-9-1, PET-PAM-PAA-7-3, and PET-PAM-PAA-5-5.**

The blue colour seen on PET and PET-PAM-10 samples could be due to attachment of TBO to the carboxylate groups in the structure of polyester and PAM. The release of TBO from the two (PET and PET-PAM-10) were negligible and considered 0. However concentration of released TBO in 50% acetic acid from PET-PAM-PAA-9-1, PET-PAM-PAA-7-3 and PET-PAM-PAA-5-5 were significantly increased ( $p < 0.05$ ,  $n = 3$ ) as shown in Figure 16 when compared to PET-PAM-10 samples. Furthermore this concentration was increased significantly ( $p < 0.05$ ,  $n = 3$ ) when the ratio of acrylic acid to acrylamide was increased. The concentration of released TBO was 0.001679924 mmol for PET-PAM-PAA-9-1, 0.003211705 mmol for PET-PAM-PAA-7-3 and 0.003972386 mmol for PET-PAM-PAA-5-5.

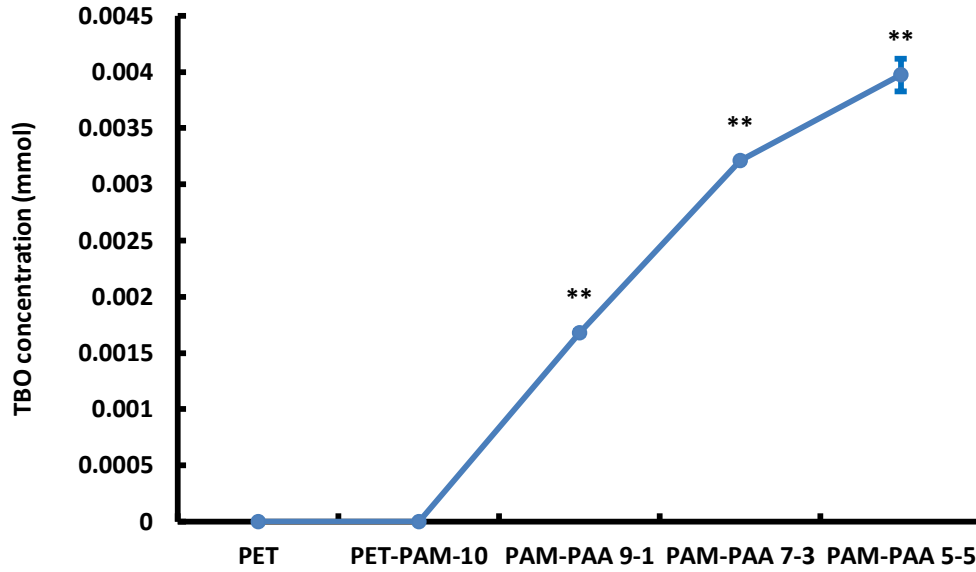


Figure 16- Concentration of released TBO in 50% acetic acid. Double asterisks indicate significant difference ( $p < 0.05$ ,  $n = 3$ ) between the value of PET-PAM-10 and other values.

Increase in the acrylic acid to acrylamide ratio caused increase in the absorption and release of dye from sample. It is possible to explain this increase by pointing out that the negative charge of samples increases after increasing the acrylic acid content in the hydrogel structure, and since TBO bears positive charge the attraction between these two opposite charge causes increase in the absorption and release rate of this dye to and from samples.

#### 4.2.1.2 Peeling energy test

One of the main purposes of this dissertation was to reduce the adherence of wound dressings to the wound bed. In the first part of this dissertation deposition of the PAM layer aided reducing the adherence of silver based and PET dressings to the gelatine cast (simulated exudates model). In the second part to aid better loading of our positive dendrimer biocide and better control its release, acrylic acid was copolymerized with acrylamide in the hydrogel structure. Here the *in vitro* gelatine cast model was similarly used to study the adherence and peeling energy of the PET-PAM-PAA samples.

As shown in Figure 17 (red columns) the required peeling energy for removal of PET-PAM-PAA dressings that are not loaded with any model drug are not significantly different ( $p > 0.05$ ,  $n=3$ ) from required energy for removal of a PET dressing. This is explainable by pointing out that portentious gelatine contains amine moieties in its structure which can be a source of positive charge. Also, since the pre-wetted samples contained ionized carboxyl group; the attraction of these two opposite charges will boost the peeling energy.

However, as explained the copolymerization of acrylic acid and acrylamide were done with the aim of better loading the positively charged biocide. Thus it is possible to propose that the negative charge of the PAM-PAA layer will be partially covered by the positively charged biocide after loading, making the hydrogel layer mostly neutral. The neutral biocide loaded dressings would be less adherent to the gelatine cast compare to the not loaded dressings. To test this hypothesis positively charged BDDAB (which contain quaternary ammonium moiety) as a model drug was loaded on the dressings via a similar post loading method that was previously used for loading dendrimer biocides on samples.

As shown in Figure 17 a significant reduction ( $P < 0.05$ ,  $n=3$ ) was be seen in the required peeling energy for the removal of the loaded dressing compare to their not loaded counterparts. Furthermore in case of samples containing higher acrylic acid to acrylamide ratio (PET-PAM-PAA-7-3 and PET-PAM-PAA-5-5) the required peeling energy for removal of gelatine cast got to a significantly lower ( $p < 0.05$ ,  $n=3$ ) value compare to the pristine PET. Although even after loading the positive drug on the samples the required peeling energy is still higher than reported peeling energy by Thomas *et al.*<sup>200</sup>, but it is possible to point out that the required peeling energy for removal of the PET-PAM-PAA-7-3 ( $626.24 \pm 106.60$  J/m<sup>2</sup>) and PET-PAM-PAA-5-5 ( $639.48 \pm 344.93$  J/m<sup>2</sup>) are smaller than the pristine PET ( $1630.96 \pm 392.76$  J/m<sup>2</sup>). Thus, making the dressing less adherent compare to the pristine PET.

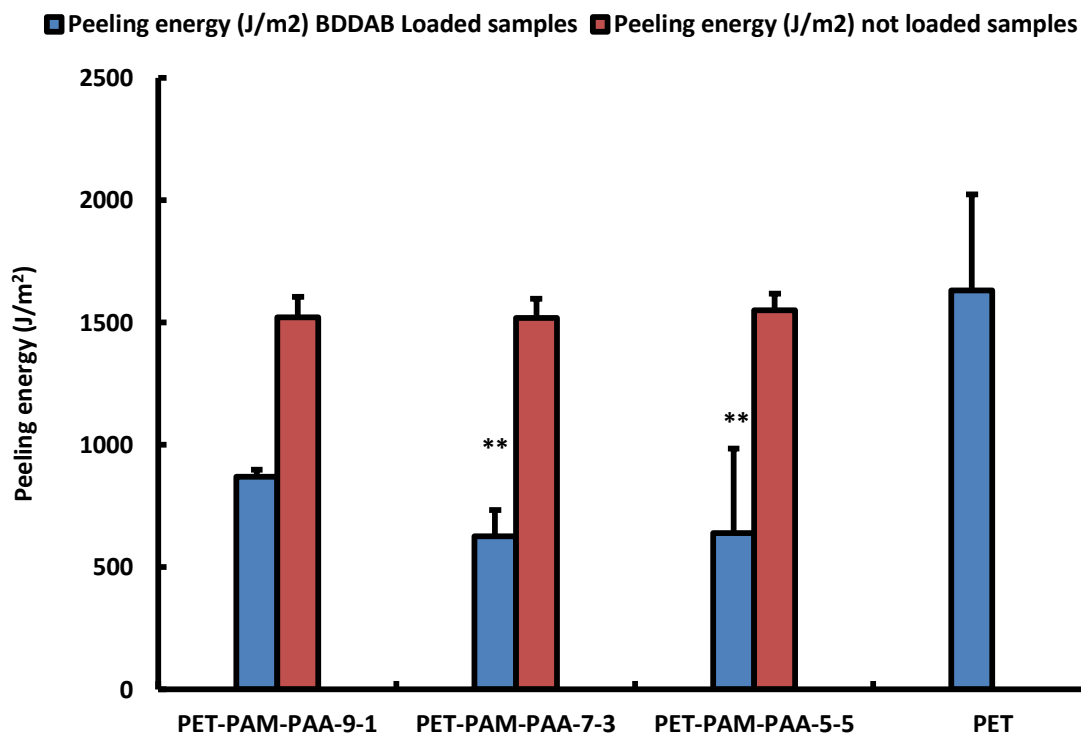


Figure 17- Comparing peeling energy of BDDAB loaded and not- loaded dressings, with PET dressing. Double asterisks indicate significant difference ( $p < 0.05$ ,  $n = 3$ ) between the peeling energy value of PET dressings and other values.

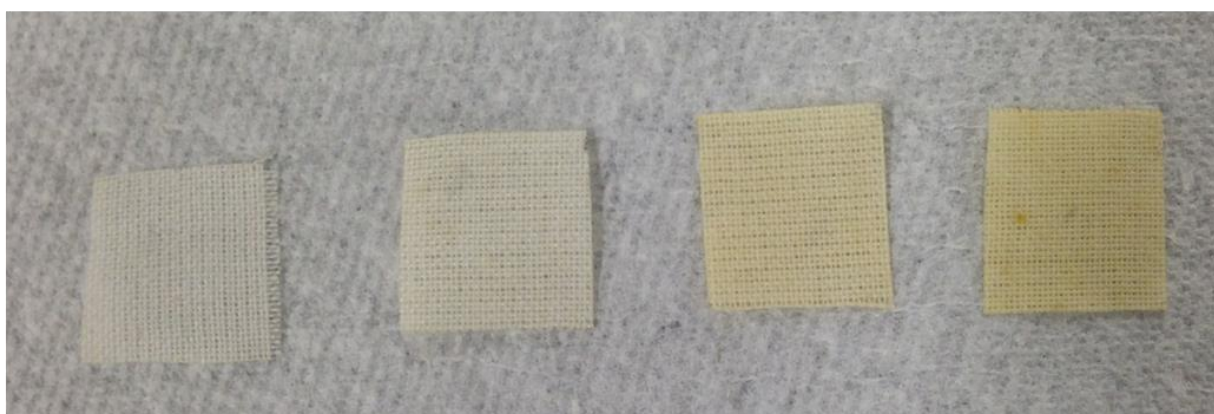
#### 4.2.2 Preparing antimicrobial dressing- Loading and release of biocide

An Antimicrobial dressing is recommended to be used for prevention or treatment of colonization or wound infection. Release of drug at the wounded site by dressing provides less bacterial resistance possibility and lower healing intervention.<sup>219</sup> Hydrogels due to their porosity and bio-compatibility are among the targeted materials and potential candidates for drug delivery and wound dressing applications.<sup>165</sup> To overcome their lack of mechanical strength in contact with water it is possible to coat them on a textile surface.<sup>219</sup> The textile will provide sufficient strength for hydrogel and hydrogel itself propose the possibility of loading drug and release it in the wound site.<sup>219</sup>

In this study poly (amidoamine) dendrimer based biocide was loaded on polyacrylamide or polyacrylamide-*co*-poly acrylic acid hydrogel-PET to be used as a wound infection control

dressing. Different molar ratio of acrylic acid and acryl amide the monomer were used to study the drug loading behaviour, release and antimicrobial ability of the dressings.

Loading drug was done via a post loading method. Dry samples were placed in 2 mL of 0.002137M biocide solution and shaken at room temperature. Sample would swell in the solution and biocide can penetrate in the porous structure of the hydrogel. Distribution of loaded drug on samples was evaluated by dividing the weight of loaded drug (mg) per weight of  $2 \times 2 \text{ cm}^2$  samples (g). As visible in Scheme 8 sample that contain higher ratio of acrylic acid to acrylamide (PET-PAM-PAA-5-5) in the right has the yellower colour compare to the other samples; the yellow tone shows the loaded biocide on samples. This tone decreases from right to left and brightest sample in the left side of the picture is the PET-PAM-10.



**Scheme 8- Loaded Biocide on samples, Left to right: PET-PAM-10, PET-PAM-PAA-9-1, PET-PAM-PAA-7-3, PET-PAM-PAA-5-5**

The negative charge of the acrylic acid owing to ionization of its carboxyl moieties in PBS (pH=7.4) aids better absorption of positively charged biocides and therefore higher loading ( $P < 0.05$ ,  $n=15$ ) of biocide can be seen on the samples containing acrylic acid (PET-PAM-PAA-9-1:  $67.73 \pm 9.57$ , PET-PAM-PAA-7-3:  $134.89 \pm 30.54$  and PET-PAM-PAA-5-5:  $138.095 \pm 16.98 \text{ mg/g}$ ) compare to the PET-PAM-10 ( $15.02 \pm 8.67 \text{ mg/g}$ ). (Figure 18)

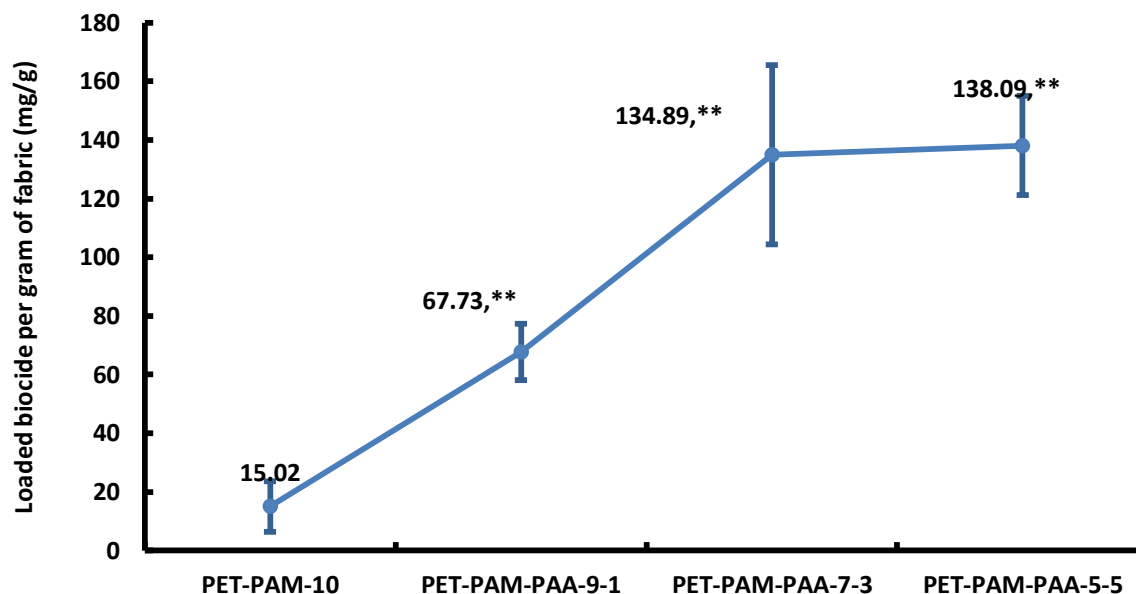


Figure 18- Weight of loaded biocide (mg) per samples weight (g). Double asterisks indicate significant difference ( $p < 0.05$ ,  $n = 15$ ) between the value of PET-PAM-10 and other values.

Release of biocide from loaded samples was investigated by placing dry samples in 10 mL PBS (0.1M, pH=7.4) solution at 32°C (skin temperature) and measuring absorbance of aliquots in specific time intervals. The pH of the PBS solution would very much simulate the pH of a burn wound according to a report by Sharpe *et al.* in which they reported pH=7.32 for adult wound that failed to heal after second dressing change.<sup>220</sup>

Upon the absorption of PBS, the hydrogel matrix will start to swell in the environment and the biocide will start releasing with initial burst release behaviour. Figure 19 shows cumulative release from hydrogel-PET samples. From day one the release was studied every 24 hours. After 24 hours of starting the test, the cumulated drug released in 10 mL PBS from PET-PAM-10 was  $1.22 \pm 0.1$  mg, which was significantly ( $P < 0.05$ ,  $n = 4$ ) lower than those of PET-PAM-PAA-9-1 ( $3.82 \pm 0.64$ mg), PET-PAM-PAA-7-3 ( $4.36 \pm 0.24$ mg) and PET-PAM-PAA -5-5 ( $3.66 \pm 0.47$ mg). The higher cumulative release in the case of samples containing acrylic acid matches with the measured ZOI from samples in which PET-PAM-PAA samples showed higher ZOI.

Release rate showed a sustained behaviour after the burst release from day 1, with the average of  $10.68 \pm 3.51 \mu\text{g/mL}$  for PET-PAM-10,  $35.51 \pm 8.7 \mu\text{g/mL}$  for PET-PAM-PAA-9-1,  $32.99 \pm 6.29 \mu\text{g/mL}$  for PET-PAM-PAA-7-3 and  $36.58 \pm 11.89 \mu\text{g/mL}$  for PET-PAM-PAA-5-5. The sustained release reached its end (to a negligible amount) after 6 days in the case of PET-PAM-10 and after 8 days for PET-PAM-PAA-9-1. Sample PET-PAM-PAA-7-3 and PET-PAM-PAA-5-5 continued the sustain release for respectively 11 and 10 days and after that the release rate decreased to a very negligible amounts.

According our unpublished data, MIC of our dendrimer biocide is lower than  $31.73 \mu\text{g/mL}$  and its CT50 is higher than  $52.88 \mu\text{g/mL}$ . The release rate of PET-PAM-PAA-9-1, PET-PAM-PAA-7-3 and PET-PAM-PAA-5-5 can be included in this interval. In the case of an improving healing wound antimicrobial dressing should be used 14 to 21 days. Although dressings are commonly changed every 1.6 days in hospital and every 2-3 days in community <sup>7,221,222</sup> some companies claim that their dressing can continue its antimicrobial activity for periods such as 7 days (Acticoat Flex 7 <sup>223</sup>) to 14 days (Mepilex <sup>224</sup>). Thus, it can be stated that our dressing also have controlled the release rate to which make it antimicrobial and less cytotoxic and the therapeutic release rate can be continued up to 12 days.

**Table 7- Release kinetics from samples**

<i>Samples</i>	<i>k</i>	<i>Diffusion exponent n</i>	<i>Correlation coefficient, R</i>	<i>minutes till the % release reaching 60%</i>
PET-PAM-10	0.9638	0.0978	0.9670	480
PET-PAM-PAA-9-1	1.0013	0.0760	0.9769	1440
PET-PAM-PAA-7-3	0.9001	0.0718	0.9769	10080
PET-PAM-PAA-5-5	0.8622	0.0750	0.9658	12960

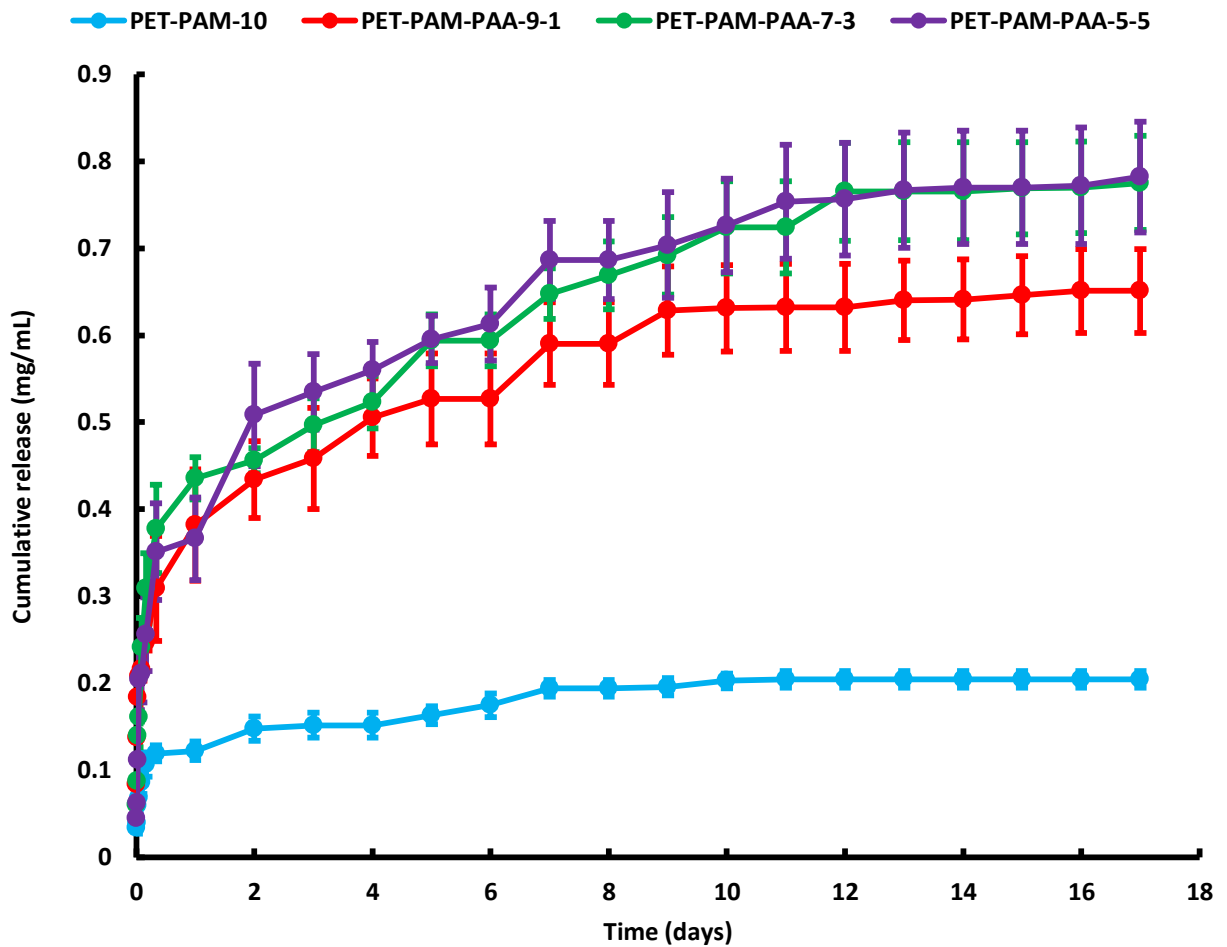


Figure 19- Cumulative release from samples (mg/mL)

The diffusion exponents ( $n$ ) were calculated according to Equation 4 and are presented in Table 7. This equation is valid for up to 60% release. Diffusion exponent for PET-PAM-10, and PET-PAM-9-1 were respectively 0.0978 and 0.076 and release reached 60% after respectively 8 hours and 1 day (Figure 20). Diffusion exponent for PET-PAM-PAA-7-3 and PET-PAM-PAA-5-5 were 0.0718 and 0.075 correspondingly and the 60% release was reached on 7 and 9 days. Since the entire resulted diffusion exponent from our hydrogel layers which can be considered as a thin swell-able film is lower than 0.5, the release mechanism is following a Fickian diffusion.<sup>175</sup>

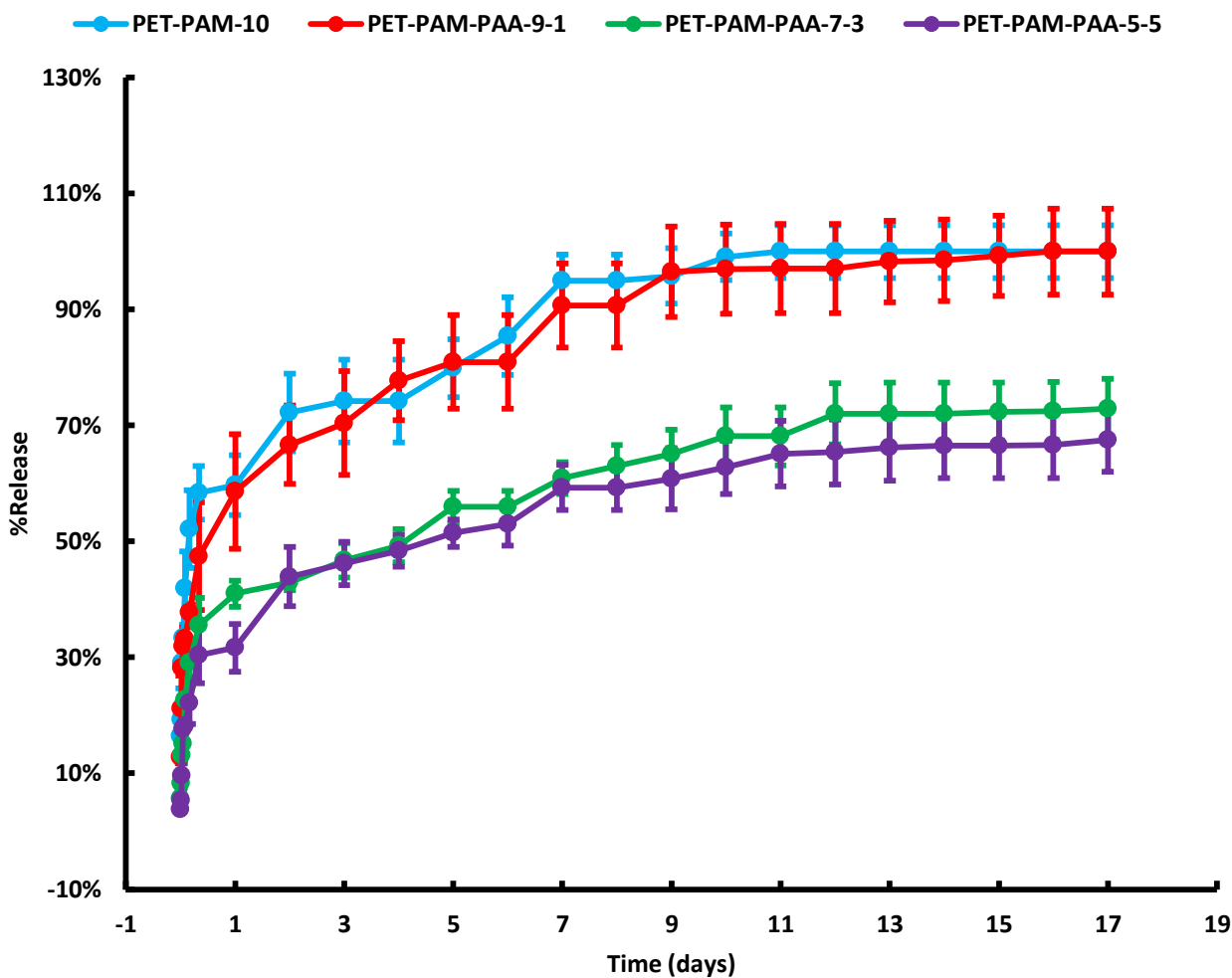


Figure 20- Release of QASG1C12 versus time curves from hydrogel deposited samples.

#### 4.2.2.1 Disk diffusion assay

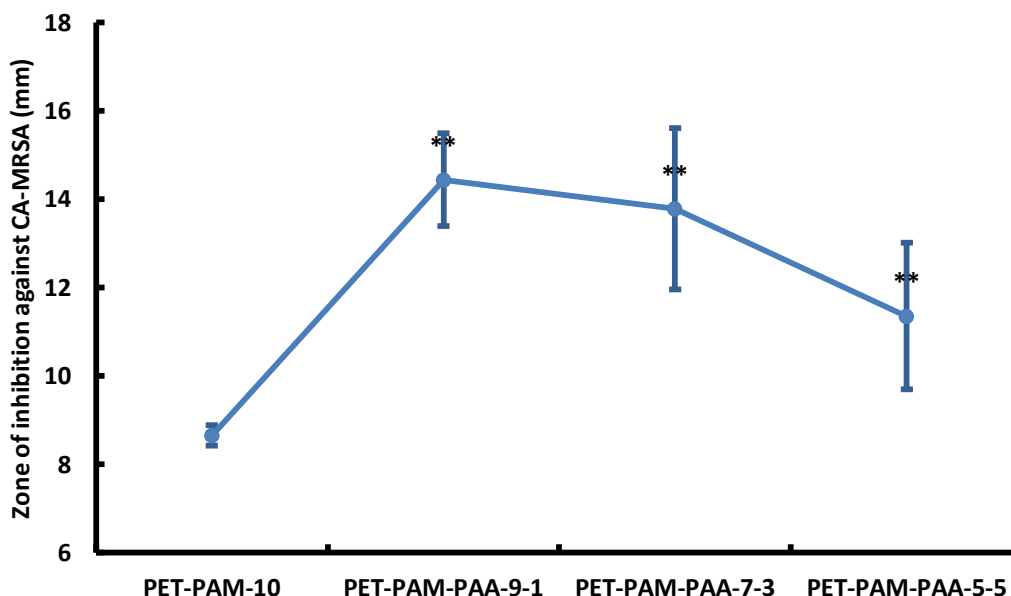
The antibacterial ability of biocide loaded samples was studied using disk diffusion assay against both CA-MRSA and MDR *P.aeruginosa*. As presented in Table 8, the diameter of inhibition zone was used as criteria to investigate the antibacterial ability of samples. Pristine PET dressings were loaded with dendrimer biocide to be considered as control. Biocide loaded samples have shown to be more biocidal against CA-MRSA compare to MDR *P.aeruginosa* (Scheme 9). Similarly Worley *et al.* stated their dodecyl quaternary ammonium generation one dendrimer has shown higher efficacy against *S.aureus* compare to

*P.aeruginosa*.<sup>225</sup> They stated this could be due to the greater physical disruption of the thicker peptidoglycan membrane in the *S.aureus*.<sup>225</sup>

**Table 8- ZOI diameter (mm) of dressings against MDR *P.aeruginosa* and CA-MRSA**

Sample	Samples' ZOI against MRSA (mm)	Samples' ZOI against <i>P.aeruginosa</i> (mm)
PET	8.35±0.12	8.37±0.28
PET-PAM-10	8.65±0.24	8.70±0.30
PET-PAM-PAA-9-1	14.43±1.05	8.83±0.28
PET-PAM-PAA-7-3	13.78±1.83	9.22±0.40
PET-PAM-PAA-5-5	11.35±1.66	8.57±0.25

Referring to Figure 21 and Scheme 9 (picture 1 and 3) a significantly larger ( $p < 0.05$ ,  $n = 6$ ) zone of inhibition was observed (mm) for biocide loaded PET-PAM-PAA dressings than biocide loaded PET-PAM-10 dressings: 14.43±1.05 (PET-PAM-PAA-9-1) and 13.78±1.82 (PET-PAM-PAA-7-3) and 8.65±0.23 (PET-PAM-10) mm against CA-MRSA.



**Figure 21- Comparing ZOI diameter of samples against CA-MRSA. Double asterisks indicate significant difference ( $p < 0.05$ ,  $n = 6$ ) between the ZOI value of PET-PAM-10 samples and other values.**

Figure 22 and Scheme 9 (picture 2 and 4) show ZOI against MDR *P. aeruginosa*, only PET-PAM-PAA-7-3:  $9.21 \pm 0.39$  (mm) showed significantly higher ( $p < 0.05$ ,  $n = 6$ ) ZOI compare to PET-PAM-10:  $8.7 \pm 0.29$  (mm). Since according to the result of t-test ZOI of both PET-PAM-PAA-9-1 and PET-PAM-PAA-7-3 are not significantly different from each other ( $p > 0.05$ ,  $n = 6$ ), It is possible to conclude that PET-PAM-PAA-7-3 have the highest antimicrobial against both bacteria.

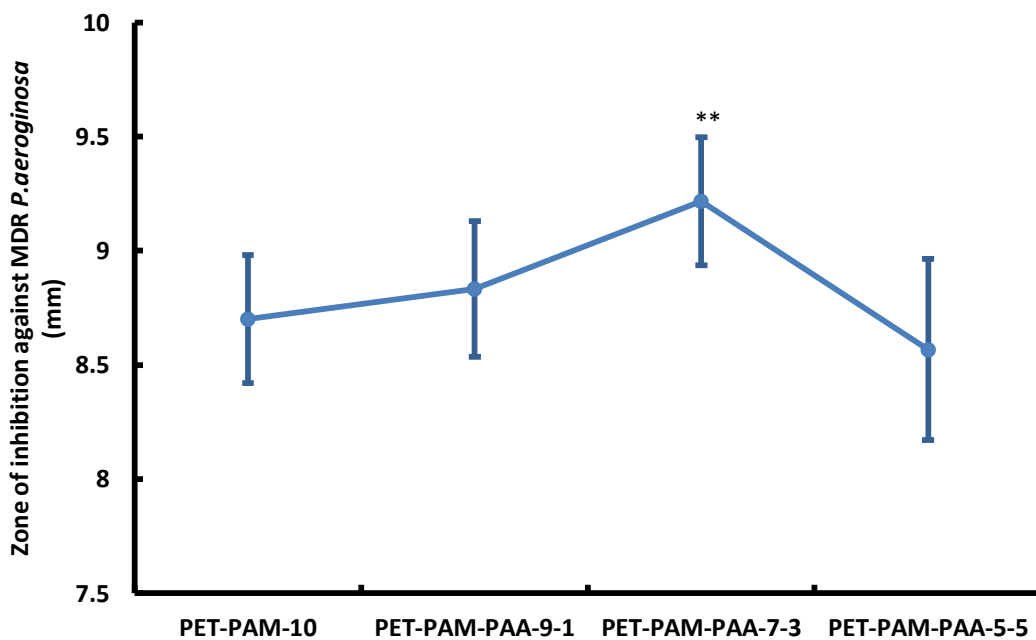
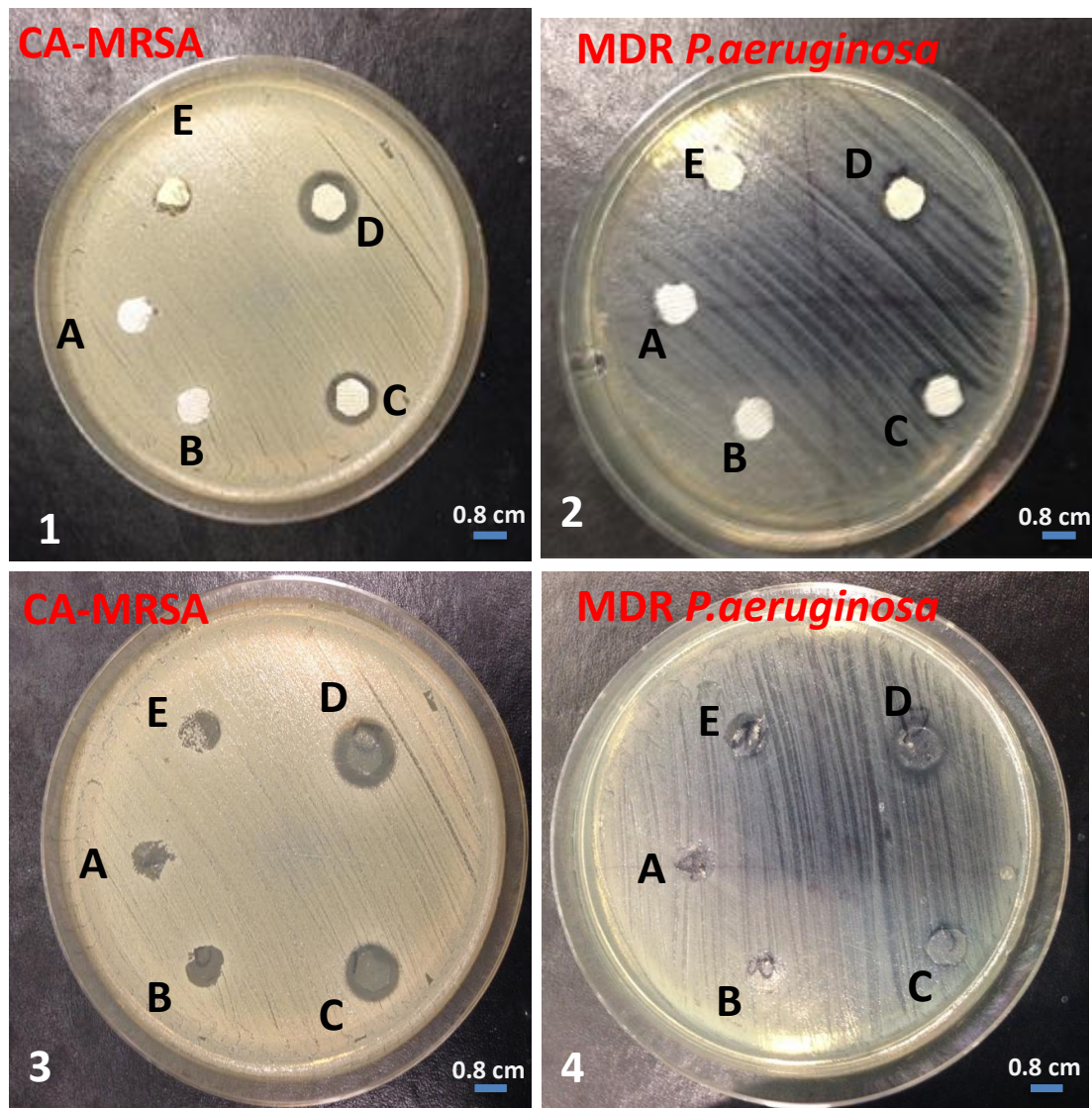


Figure 22- Comparing ZOI diameter of samples against MDR *P.aeruginosa*. Double asterisks indicate significant difference ( $p < 0.05$ ,  $n = 6$ ) between the ZOI value of PET-PAM-10 samples and other values.



Scheme 9- Pictures on the left (1 and 3) show the ZOI diameter of samples on agar plate when samples were tested against CA- MRSA and pictures on the right (2 and 4) show the ZOI diameter of samples on agar plate when samples were tested against MDR *P.aeruginosa*. A: PET, B: PET-PAM-10, C: PET-PAM-PAA-9-1, D: PET-PAM-PAA-7-3, E: PET-PAM-PAA-5-5. (Sample E in picture 1 and 3 was wrinkled) Also ZOI of the dressings after removing them from agar are visible on picture 3 and 4.

***Chapter 5***  
***Summary and Conclusion***

## CHAPTER 5. SUMMARY AND CONCLUSION

Although the improvements in post-burn care managements have decreased the rate of mortality in the burned patients recently; the infection of burned wound is still one of the common causes in post-burn mortality.<sup>19,226</sup> Utilization of burn-wound dressings assists protecting wounded area from microorganism invasion, and preventing heat loss from the body.<sup>30</sup> Important criteria for a wound dressing are proper absorbency and moisture retention, being atraumatic, and having antimicrobial ability. A dressing with combination of all these characteristics is still not clinically available.<sup>14</sup> Efforts to fill this gap can be divided in two strategies: (1) Introducing a method to modify the current available dressings in the market or (2) Looking for a method to achieve all these necessities in one proper dressing by presenting a brand new wound dressing.

In the first part of this dissertation we aimed to utilize the promising characteristic of hydrogel to improve the common existing Ag-nanoparticle-based-antibacterial burn-wound dressings. A UV-radiation grafting method was applied to modify clinically existing silver dressings (Acticoat Flex 3 and Silverlon) with acrylamide-hydrogel since the low-adherent obtained silver-dressing would be more desired for future clinical usage.

According to our results, using pressure during hydrogel deposition could reduce the hydrogel swelling while maintaining the peeling-energy at a constant level. Loading the polyacrylamide-layer on the commercial silver-based samples reduced their adherence to the simulated-exudate model to less than 300-400 J/m<sup>2</sup> which was previously reported to be suitable peeling-energy.<sup>200</sup>

The deposition of this polyacrylamide layer did not significantly compromise the antimicrobial ability of the samples against common burn-wound infecting bacteria (CA-MRSA and MDR *P.aeruginosa*). Furthermore, edges of the samples were also coated via the PAM layer to confirm that the uncompromised antibacterial ability is not due to the leaked

silver from the edges of samples and similar results were obtained. Cationic-silver release from Acticoat-samples was studied via ICP-OES. Results showed that cumulated silver released from treated and untreated samples in 48 hours were not significantly different from each other. Results from cytotoxicity tests of treated and untreated silver-based dressings against both fibroblast and keratinocyte showed no reduction in the viability of cells after deposition of PAM layer.

Effect of autoclave sterilization method was studied on adherence and antimicrobial properties of the dressings. It was observed that this method compromises the bactericidal ability of dressings and increases their adherence. Thus ethylene oxide sterilization method was tested and suggested as a replacement sterilization method.

Since the first aim of the dissertation was achieved and the PAM layer reduced the wound adherence of the available silver dressing without compromising the antimicrobial ability of the dressing or increasing the toxicity of these dressings to the tested cells; it is recommended for to further carry out this study on the animal model level.

In addition of silver and other antibacterial agents, quaternary ammonium compounds are attractive cationic-agents with a hydrophilic and a hydrophobic site. They are known to be biocidal compounds.<sup>227</sup> In contrast to many other biocides these compounds retain their properties after application.<sup>228</sup> Adding long alkyl chains increases the biocidal ability of these compounds.<sup>225</sup> Recently, a quaternary-ammonium-based biocide, prepared in our group, have shown promising antibacterial ability against CA-MRSA and MDR *P.aeruginosa*. Furthermore, the MIC concentration of this compound against MDR *P.aeruginosa* was reported to be lower than its CT50 concentration.

As the second part of this dissertation, the first generation polyamidoamine dendrimers linked with long chain (12 carbons) quaternary ammonium biocide was incorporated to and from hydrogel-deposited PET-dressings. The aim was to achieve a dressing with all desired

abilities of an ideal burn-wound dressing by reaching a sustained controlled release of this cationic biocide.

To achieve a better loading and a controlled release of this positively charged biocide, a combination of acrylic-acid and acrylamide were copolymerized and deposited on the PET dressing via the UV-radiation grafting method that was utilized in the first part of this project. Concentration of acrylic-acid was determined via titration of carboxyl groups after attaching them to the positive-TBO dye. As expected, samples containing higher acrylic-acid to acrylamide ratio showed higher carboxyl group concentration. PET-PAM-PAA samples loaded with model drug showed lower peeling energy in comparison to the pristine PET. Positive biocide was loaded on samples using a post-loading method and by immersion of the dry samples in biocide-PBS (pH=7.4, 0.1M) solution. Higher biocide was loaded on the acrylic-acid containing hydrogel-PET samples due to the negative charge of the hydrogel-layer at the loading pH. Results of the release studies from the biocide loaded samples showed it is possible to get a sustained therapeutic dose from the acrylic-acid-co-acrylamide dressings for more than 5 days.

After conducting disk diffusion assay against CA-MRSA and MDR *P.aeruginosa*, Results showed the biocide loaded dressings are more effective against CA-MRSA compare to MDR *P.aeruginosa*. Overall, by considering biocide release and antimicrobial and peeling energy results, it is possible to choose PET-PAM-PAA-7-3 dressings as the proper dressings to reach the aim of antimicrobial, low-adherent dressing with proper therapeutic release dose.

Although the loaded PET-PAM-PAA-7-3 dressing have shown to be less adherent to gelatin model compare to the pristine PET the adherence of this dressing to gelatin model is still higher than 300-400 J/m<sup>2</sup> which is reported as a suitable level by Thomas *et al.*<sup>200</sup> Thus, more attempts can be recommended to be made for treatments of the PET-PAM-PAA-7-3 to improve the adherence level of this dressing. A suggestion is to deposit a second layer of

polyacrylamide hydrogel on this dressing similar to what has been done in the first part of this project. Also it is recommended to load other positively charged antimicrobial agents on PET-PAM-PAA-7-3 dressing and study the possibility of using this dressing as a reservoir for other positively charged antimicrobial agents.

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