PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE
ESCUELA DE INGENIERIA

EFFECT OF BACTERIAL CONTAMINATION IN DEGRADATION OF TYROSINE-DERIVED POLYCARBONATES

DANIELA C. SOTO

Thesis submitted to the Office of Research and Graduate Studies in partial fulfillment of the requirements for the Degree of Master of Science in Engineering

Advisor:
LORETO M. VALENZUELA

Santiago de Chile, June, 2015

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Members of the Committee:

LORETO M. VALENZUELA

WENDY FRANCO

ANGEL LEIVA

LUCIANO CHIANG

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"Education is a progressive
discovery of our own ignorance."

Will Durant

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LISTS OF ABBREVIATIONS

DSC	Differential scanning calorimetry
E0000	Poly(DTE carbonate)
E1001(1K)	Poly(DTR-*co*-10%-DT-*co*-01%-PEG_{1000} carbonate)
M_w	Molecular weight
P. aeruginosa	*Pseudomona aeruginosa*
P(DL)LA	Poly(D,L-lactic acid)
PLGA	Poly(glycolic acid)
S. aureus	*Staphylococcus aureus*
SEC	Size exclusion chromatography
SEM	Scanning electron microscopy
T_g	Glass transition temperature
TGA	Thermogravimetric analysis
WU	Water uptake

RESUMEN

La infección de implantes es un problema clínico significativo, que puede generar el rechazo del implante o el colapso prematuro del dispositivo implantado. Los polímeros son ampliamente utilizados en distintas aplicaciones médicas debido a sus propiedades ventajosas, como por ejemplo su biocompatibilidad y biodegradación ajustables, pero para ser utilizados en aplicaciones clínicas es necesario caracterizar su comportamiento en un ambiente contaminado. Los terpolímeros de policarbonato derivados de tirosina son un nuevo tipo de polímeros que parecen prometedores para varias aplicaciones biomédicas. Sin embargo, su comportamiento en caso de infección aún no ha sido descrito. En este trabajo estudiamos el efecto de la contaminación bacteriana en la degradación de terpolímeros, poliácido láctico-*co*-glicólico (PLGA) y poliácido D,L-láctico (P(DL)LA), así como la capacidad de las bacterias para proliferar y formar biopelículas en la superficie del polímero. Para lograr esto, muestras de películas moldeadas por compresión fueron incubadas en dos medios no estériles, tampón fosfato salino (PBS) y PBS con fibrinógeno, promoviéndose la contaminación ambiental con *Pseudomona* sp. Adicionalmente, muestras de terpolímeros y P(DL)LA fueron incubadas en PBS estéril inoculado con *Staphylococcus aureus* resistente a meticilina, *Pseudomona aeruginosa* y *Pseudomona* sp. ambiental. Ambas especies de *Pseudomona* fueron capaces de sobrevivir al marco experimental, mientras que *S. aureus* no fue viable después de un día de incubación. La tasa de degradación de los terpolímeros fue incrementada por la presencia de *Pseudomona* sp. ambiental en el medio no estéril, pero la degradación de PLGA y P(DL)LA permaneció constante. No se observaron cambios en la degradación de los polímeros incubados en medio estéril inoculado con bacteria, probablemente debido a la baja concentración bacteriana inoculada. Sin embargo, se observó que las especies de *Pseudomona* fueron capaces de adherirse a la superficie, secretando sustancias para formación de biopelículas. Estos resultados muestran que la infección de implantes de terpolímeros, ya sea antes o después de la implantación, puede llevar a degradación acelerada del dispositivo y a la formación de biopelículas que agravan la infección.

ABSTRACT

Implant infection is a significant clinical problem that can cause implant rejection and premature collapse of the implanted device. Polymers are widely used in several biomedical applications due to their advantageous properties such as biocompatibility and adjustable biodegradability, but in order to be used in clinical applications it is necessary to characterize their behavior in a contaminated environment. Tyrosine derived polycarbonates terpolymers are a new type of polymers that seem promising for several biomedical applications. However, their behavior in case of infection has not yet been described. In this work, we studied the effect of bacterial contamination in degradation of those terpolymers, poly(lactic-*co*-glycolic acid) (PLGA) and poly(D,L-lactic acid) (P(DL)LA), as well as the capability of bacteria to proliferate and form biofilms over the polymer surface. To accomplish this, compression molded film samples were incubated in two non-sterile media, phosphate buffered saline (PBS) and PBS with fibrinogen, promoting environmental contamination with *Pseudomona* sp. Additionally, terpolymers and P(DL)LA samples were incubated in sterile PBS inoculated with clinically relevant methicillin-resistant *Staphylococcus aureus*, *Pseudomona aeruginosa* and environmental *Pseudomona* sp. Both *Pseudomona* species were capable to survive to the experimental setting, while *S. aureus* was not viable after one day of incubation. Degradation rate of terpolymers was increased by the presence of environmental *Pseudomona* sp. in the non-sterile medium, but degradation of PLGA and P(DL)LA films remained constant. No changes in degradation were observed in polymers incubated in sterile media inoculated with bacteria, likely due to low bacterial concentration inoculated. However, it was observed that *Pseudomona* species were capable to adhere to the polymer surface, secreting substances forming biofilms. These results show that infection of polymer implants, either before or after implantation, could lead to accelerated degradation of devices and biofilm formation that worsen the infection.

Keywords: polymer degradation, implant infection, microbial contamination, polycarbonates, PLGA, PLA.

1. INTRODUCTION

1.1 Polymers in biomedical applications

Biomaterials are widely used in several biomedical applications. Nowadays they are being used as sutures, heart valves, pacemakers, vascular stents, drug delivery systems and, more recently, as scaffolds for bone, dental, cardiovascular and soft tissue engineering. Millions of patients have received medical implants which saved and improved their life quality and it is estimated that biomaterials market for implantable devices exceeded 250 billion dollars in 2014 (Bryers, Giachelli, & Ratner, 2012).

A biomaterial is defined as a material intended to interact with biological systems (Ratner, Hoffman, Schoen, & Lemons, 2004). It encompasses a wide range of materials, both resorbable and non-resorbable. Examples of biomaterials are ceramics, glasses, metals, hydrogels and polymers.

Polymers are the larger class of biomaterial. They could be obtained from natural sources – such as cellulose, chitosan, natural rubber or even DNA – or synthetically made by organic processes. In the last decade biodegradable synthetic polymers have received much attention, because their advantages over other materials. They can be designed to tune the exact needs of a certain application, for example, they could have a specific drug release profile or a precise degradation kinetic that match the regeneration rate of the damaged tissue, as well as adequate mechanical properties to imitate tissue properties (Gunatillake & Adhikari, 2003; Kohn, Welsh, & Knight, 2007). Additionally, they can be fabricated into shapes that promote host cells integration or even they can be modified with chemical functional groups. However, as a downfall they have been related with low biocompatibility, poor processability, loss of mechanical properties during degradation and release of acidic degradation products that may trigger an immune response (Gunatillake & Adhikari, 2003).

Synthetic polymers encompass a wide range of characteristics. For example, silicone rubber, polyethylene (PE), polypropylene (PP) and poly(methyl methacrylate) (PMMA) are hydrophobic and non-water absorbing materials, while poly(vinyl chloride) (PVC),

poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and copolymer poly(lactic-*co*-glycolic acid) (PLGA) are more polar materials. Poly(hydroxyethyl methacrylate) (PHEMA) swells in the presence of water, while poly(ethylene glycol) (PEG) is highly hydrophilic and water soluble (Ratner et al., 2004).

The U.S. Food and Drug Administration (FDA) has approved specific devices and implants made with synthetic polymers. It is important to notice that FDA does not approve polymers itself, instead it approves each particular polymer application. Currently approved devices include, among others, sutures of PGA, PLA, PLGA, polycaprolactone (PCL) and polydioxanone (PDS); drug delivery systems of polyanhydrides containing sebacic acid, and a hernia repair device of tyrosine-derived polyarylate (Kohn et al., 2007).

Despite several research efforts, the biomaterial community have found difficulties to move from simple poly(hydroxy acids), such as PLA, to more complex bioactive materials that can address complex interactions with the host cells while fulfilling its intended performance (Kohn et al., 2007). This have been a major drawback in the development and growth of tissue engineering, which aims to implant a temporary scaffold that integrates with the host cells, allowing the body to heal itself. Kohn et al. suggested that the combination of experimentation with combinatorial approaches and computational modeling will help the biomaterial community to overcome the complexity of biological systems and therefore develop more complex materials. Following this principles, Kohn's research group developed tyrosine-derived polyarylates (Brocchini, James, Tangpasuthadol, & Kohn, 1998) and tyrosine-derived polycarbonates (Pulapura & Kohn, 1992) combinatorial libraries. The first family has applications already approved by the FDA, like a hernia repair device. The second family has shown promissory performance for several applications and it will be discussed in detail later in this document.

To select a biomaterial for a certain application, relevant properties must be considered. These includes: rate of degradation within the time frame required of the application, degradation mechanism and the release of non-toxic products, appropriate mechanical properties - which in case of tissue engineering scaffolds, must be maintained during most

part of the tissue regeneration process - and processability, with shapes and porosity that support and promote cell attachment and proliferation (Gunatillake & Adhikari, 2003; Kohn et al., 2007). All of the above must be reached without triggering and adverse host immune response that could lead to the biomaterial rejection. There are several parameters influencing these properties, such as chemical nature, molecular weight, molecular weight distribution, and processing methods.

As mentioned above, degradation plays a key role in the performance of biodegradable scaffolds. According to Mainil-Varlet et al. (Mainil-Varlet et al., 2001) factors involved in degradation of bioresorbable polymers are, among others, chemical composition, molecular weight distribution, crystallinity, chain mobility, surface-to-ratio volume, shape, dimensions, porosity, hydrophilicity and hydrophobicity.. Also, the environmental characteristics are relevant, such as pH, ionic strength, temperature and buffering capacity.

There are four major mechanisms of degradation of polymeric devices in the body: hydrolytic, oxidative, enzymatic and mechanical (Kaali, Strömberg, & Karlsson, 2011). Hydrolytic degradation begins with water absorption, which initiates reactions between water molecules and polymer chain, which diminishes molecular weight. The degradation process could promote further water uptake and, in some cases, it could accelerate and catalyze degradation. Oxidative degradation occurs inside the body and it is catalyzed by peroxides produced by macrophages, in order to break down the material and eliminate it from human body. Enzymatic degradation also occurs inside the body and it is related to the activity of tissues and cells, as well as microorganisms. Depending on the type of enzymes produced two kind of enzymatic degradation could take place: oxidation or hydrolysis. Besides chemical degradation, polymers could also suffer physical and mechanical degradation due to swelling and friction.

Despite having good mechanical properties or certain degradation rate, a material could not be used in medical applications if it is not biocompatible during all the application lifetime. Biocompatibility is defined as the ability of the material inside the body to perform its intended function without triggering an adverse immune response (Pavithra & Doble, 2008).

Biocompatibility is mainly a surface phenomenon, depending on polymer-protein, polymer-cell and cell-cell interactions. The material biocompatibility capacity is influenced by the type of material, the site of implantation, the contact duration, and the genetic pool of the host. In terms of the type of material, not only the chemical composition is relevant, but also its shape, size, roughness, porosity, degradation byproducts and sterilization method.

Resistance to bacterial colonization is also a key feature of biocompatible materials. Generally, bacteria interact with surfaces through a "conditioning film" mostly formed by proteins that adsorb onto material surface only seconds after implantation (Ratner et al., 2004). In general, surfaces that resist protein adsorption will also resist bacterial colonization. However, this kind of surfaces will also had a poor integration with host cells, since they also interact with the protein layer (Bryers et al., 2012). Polymeric device contamination, mechanisms of infection, bacteria involved and their influence over polymeric properties will be further discussed in the following sections.

1.2 Implants bacterial contamination

Implant infection is a significant clinical problem even today. Despite nowadays the majority of the implants do not get contaminated; there is still a percentage of implants that suffer of bacterial colonization. The probability of infection depends on the surgical site and procedure, but in cases of big trauma this probability is higher (Hickok & Shapiro, 2012). It is considered that approximately 10% of the implants used in bone fractures defects suffer from some kind of bacterial contamination (Johnson, Lehman, & García, 2014).

Implants contamination can take place previous implantation in case of trauma, in the surgical procedure, or latter by hematogenous spread of bacteria (Hickok & Shapiro, 2012). The former occurs when transient bacteremia (low number of bacterial cells in the blood not cleared by the immune system) successfully colonized the implant surface. Commonly this low quantity of bacteria is not capable to trigger an infection in normal conditions, but the presence of a foreign body significantly diminishes the number of bacteria needed to develop an infection inside the body (Mainil-Varlet et al., 2001).

1.2.1 Bacterial adhesion and biofilm formation

The first event to take place in infection development is the adhesion of bacteria to the polymer surface. The adhesion of bacteria depends on the properties of the material, type of bacteria and the environment. Accordingly to Pavithra and Doble (Pavithra & Doble, 2008), key polymer properties influencing bacterial attachment are chemical composition, surface charge, hydrophobicity, surface roughness and physical configuration. In the other hand, microbial characteristics relevant for adhesion are surface charge, present of flagella or fimbriae and extra-polysaccharide coat. Finally, the environmental factors associated with bacterial adhesion are medium temperature, pH, concentration of electrolytes, time of exposure, and concentration of bacteria.

In a time lapse from seconds to minutes after implantation, host molecules such as fibronectin, vitronectin, fibrinogen and other proteins, glycoproteins, polysaccharides, and lipids are adsorbed to the material surface, forming a conditioning film (Padera, 2006). This implies that cells and microorganisms arriving at the implant surface will have to interact not only with the biomaterial itself, but also rather with this conditioning layer. In normal conditions, proteins in the conditioning film aid tissue remodeling by recruitment of cells. However, in contaminated environments, some of these proteins also interact with microorganisms through hydrophobic, van der Waals or electrostatic interactions, allowing bacteria to reversible adsorb to the material. Notwithstanding, some bacteria have surface molecules that allow them to directly attach to non-covered polymer surfaces (Padera, 2006).

Serum proteins in the conditioning film could promote or inhibit the bacterial adhesion. Fletcher et al. (Fletcher, 1976) observed that bovine serum albumin (BSA), gelatin, fibrinogen and pepsin inhibit the adhesion of marine *Pseudomona sp*. on polystyrene. In a later study, Kuusela et al. (Kuusela, Vartio, Vuento, & Myhre, 1985) showed that *Staphylococcus aureus* attachment is promoted in glass slips covered with fibrinogen and fibronectin. However, in the case of streptococci, only fibrinogen promotes the adhesion, while fibronectin appears to have a weak effect. Accordingly, Herrmann et al. (Herrmann et

al., 1988) observed that fibrinogen promoted the adherence in polymethylmethacrylate coverslips of *S. aureus* strains and some coagulase-negative staphylococci and that fibronectin promotes the adherence of all the strains, probably due to a specific, receptor-mediated process. Additionally, they observed that albumin inhibit almost completely the adhesion of all bacteria to the polymer surface, probably due to a decreased hydrophobicity in the surface. Considering the above, the effect of protein adsorption in bacterial adhesion seems to be highly dependent of material, type of bacteria and nature of the protein, and must be assess case by case.

It has been observed that degradable polymers PLA and poly(orthoester) (POE), attached the same amount of bacteria that non-degradable polymers such as PE (Barton, Sagers, & Pitt, 1996). The authors suggested that degradation did not interfere with bacterial attachment possibly because multiple bacteria-polymer contact points are involved in bacterial adhesion. Additionally, for polymers with acidic degradation byproducts, such as PLA, it has not been observed that the degradation process and the consequent drop in pH affect bacterial attachment. (Mainil-Varlet et al., 2001).

The amount of available nutrients in the culture media also affects the bacterial adhesion to a polymer surface. Barton et al. (Barton et al., 1996) observed that almost twice of bacteria attach to a polymer surface immersed in phosphate buffer saline (PBS) than in tryptic soy broth (TSB). The same was observed by Costerton et al. (Costerton, Stewart, & Greenberg, 1999). It has been postulated that surface attachment helps bacteria to proliferate in nutrient-poor environments because nutrient availability increase around the polymeric surface (Fletcher, 1976).

Once attached, the microorganisms start to proliferate and biofilm formation could take place. Biofilms are aggregates of one or several microorganisms, formed by attachment of microbial cells to the host surface (Kaali et al., 2011). Biofilms layers are 50-100 µm thickness and they are composed of 73-98% extracellular material and void spaces (Pavithra & Doble, 2008). This adhesive matrix is called exopolysaccharide (EPS), which enhance the adhesion of microorganisms to the surface and protect the biofilm from the environmental

threats. In particular, biofilm formation protects the bacteria from antibiotics – due to low antibiotic diffusion in EPS and limit effect against not growing bacteria, commonly present in biofilms – and from host immune response (Kaali et al., 2011). Also, biofilms environments are suitable to develop antibiotic resistance strains, due to the high availability and interchange of DNA between bacterial species.

Gristina et al. (Gristina, 1987) suggested that host cells and pathogens are in a "race to the surface" of the implant. When host cells arrive first, they start the tissue remodeling and their adhesion to the implant surface decrease the probability of biofilm formation. Correspondingly, when bacteria colonize first the implant surface, it limits the host cell population and tissue remodeling is hindered. Therefore, in early implants contamination (when the pathogen is in the implant or in the surgical site) the absence of host cells adhered to the implant increase the probability of successful bacteria colonization.

1.2.2 Bacteria involved in implant infections

Several bacteria have been associated with implants infections. In orthopedics, the most common microorganisms involved in infections are gram-positive bacteria, being especially prevalent *S. aureus* strains (Hickok & Shapiro, 2012). On the other hand, *Pseudomona aeruginosa* have been identified as the most frequent gram-negative bacillus associated with infection of foreign body implants and other medical devices (Brouqui, Rousseau, Stein, Drancourt, & Raoult, 1995). *P. aeruginosa* also could develop antibiotics resistance, making it a significant clinical pathogen (Johnson et al., 2014).

A wide identification of microorganisms involved in bone infections was conducted by Khosravi et al. (Khosravi, Ahmadi, Salmanzade, Dashtbozor, & Montazeri, 2009). The authors isolated bacteria from bone implant infections of 165 patients in order to describe the bacteriology of these infections. They found that *S. aureus* was the most prevalent bacteria, present in more than 20% of the infections. The second most frequent microorganism was *Klebsiella ozaenae*, which was present in about 16% of the infections, followed by *P. aeruginosa*, responsible for 15% of the infections approximately. Other

microorganisms isolated in smaller quantities were *Escherichia coli* and *Staphylococcus epidermidis*.

Although bacteria are the predominant contamination agent, other microorganisms have been linked to implant contamination. For example, in infections of ventricular assist devices, in addition to *S. aureus* and *P. aeruginosa*, fungi *Candida* sp. have also been identified as a relevant pathogen (Padera, 2006).

1.2.3 Effects of bacteria in polymer degradation

As previously mentioned, degradation of implants could be accelerated by enzymatic activity. These enzymes could be released by host cells, but also from bacteria present at the implant site. This implies that the presence of bacteria may accelerate device degradation (Kaali et al., 2011) increasing the risk of premature collapse and failure of the implant (Hofmann, Liedtke, Ruckdeschel, & Lob, 1990).

The effect of bacterial contamination in PLA degradation was assessed *in vitro* by Hofmann et al. (Hofmann et al., 1990). They studied the effect of several clinically relevant bacteria - *S. aureu*s, *S. epidermidis, E. coli* and *P. aeruginosa*, and the fungi *Candida albicans* - incubated in TSB, in the degradation rate of PLA. They observed that no significant difference in microorganism growth was trigger by the presence of PLA or its degradation byproducts. Also, the presence of PLA did not affect the pH of the sterile or contaminated medium during the timespan of the experiment. The most important finding of that study was that no change in PLA degradation was observed in presence of contaminated culture media with those microorganisms.

Later, Mainil-Varlet et al. (Mainil-Varlet et al., 2001) studied the effect of *in vivo* bacterial presence in degradation and mechanical properties of two polylactide implants with different degradation rates, P(L)LA and P(L/DL)LA, which degrades faster than the first. They inserted for 4 weeks extruded rods of both polymers in the medullary cavity of rabbit tibiae previously inoculated with *S. aureus.* They observed that bacteria did not affect either the

mechanical properties, such as bending strength, bending modulus and shear stress, or molecular weight. Additionally, no significant difference in the incidence of local infection was observed between polylactides, which means that polymer degradation rate and the corresponding decrease in environmental pH may not affect bacterial adhesion. However, the number of bacteria that elicit a local infection in these polymer were 10 fold lower than those needed in metal implants, which means that polylactides may have an increased risk of infection. In that study, bone growth did not seem to be affected by the presence of bacteria or the existence of bone infection.

Unlike the above, in an *in vivo* study performed by Johnson and coworkers (Johnson et al., 2014), reported that *P. aeruginosa* infection (5.0×10^5 CFU/mL) on a PEG hydrogel implant functionalized with a growth factor (BMP-2) intended for critically-sized radial segmental defect in mouse, significantly decrease the healing rate of the damaged bone in comparison with the non-contaminated implant. Molecular weight analysis showed that *P. aeruginosa* did not trigger changes in degradation rate of the polymer, therefore the healing potential decrease of the implants was not caused by changes in degradation. It was observed that after 14 days of incubation bacteria was also present in the polymer device, in a concentration only one order of magnitude lower (2.5×10^4 UFC/ml), which suggest that *P. aeruginosa* could be forming biofilms that protect them from host immune response. These results agree with the current knowledge that bacterial cells and host cells "compete" to attach to the surface; if bacterial cells attach first, they hinder the adhesion probability of host cells and therefore difficult tissue remodeling.

The effect of microbial enzymes over a polymer is closely related with the effect caused by enzymes released by host cells. Several researchers have studied the difference between *in vitro* and *in vivo* degradation of polymers intended for biomedical applications in order to assess the effect of the enzymes released by host cells over its degradation process. For some polymers, degradation *in vivo* is accelerated either by enzymes or changes in pH induced by these cells (Holy, Dang, Davies, & Shoichet, 1999). In the case of polylactides, several scientist had found no significant difference between i*n vitro* and *in vivo* degradation (Bos et al., 1991; Hooper, Macon, & Kohn, 1998; Leenslag, Pennings, Bos, Rozema, & Boering,

1987), suggesting the absence of the enzymatic involvement in the polymer degradation in the body. However, Mainil-Varlet et al. (Mainil-varlet, Curtis, & Gogolewski, 1997) observed a faster *in vivo* degradation for P(L)LA, P(L/D)LA and P(L/DL)LA. Similarly, for PLGA it has been reported either equal degradation in both scenarios (Anderson & Shive, 2012) or faster degradation *in vivo* (Lu et al., 2000; Oh, Kang, & Lee, 2006). Additionally, William et al. (Williams & Mort, 1977) showed that while abiotic hydrolysis is the major degradation mechanism in poly(hydroxy acids) such as PLA or PGA, the presence of tissue enzymes (pronase, proteinase K and bromelain) accelerated the degradation rate.

Considering the above, there is no evidence of an acceleration of degradation rate due to enzymatic activity of key bacterial pathogens such as *S. auereus* or *P. aeruginosa* in important polymers intended for biomedical applications. However, some authors have observed differences in *in vivo* and *in vitro* degradation of these polymers, likely caused by host cells enzymes.

1.3 Tyrosine Derived Polycarbonates Terpolymers

Tyrosine-derived polycarbonates are a class of synthetic polymers that belong to a family of pseudo-poly(amino acids) developed and characterized by Kohn and coworkers (Ertel & Kohn, 1994; Pulapura & Kohn, 1992). These polymers, particularly poly(DTE carbonate), have shown good biocompatibility and tunable mechanical, thermal and degradation properties, which make them suitable for biomedical applications. Despite the above, erosion and complete resorption of poly(DTE carbonate) implants are too slow for most practical application (Magno et al., 2010). For this reason, several efforts have been done to accelerate the degradation rate of the tyrosine-derived polycarbonates, synthetizing copolymers with poly(ethylene glycol) (PEG) (Yu & Kohn, 1999; Yu, Mielewczyk, Breslauer, & Kohn, 1999) or, in other approach, synthetizing copolymers with desaminotyrosil-tyrosine (DT) (Abramson, 2002).

Recently, Magno et al. (Magno et al., 2010) synthetized and characterized tyrosine-derived polycarbonate terpolymers, generated by random copolymerization of the L-tyrosine derived

monomers desaminotyrosyl-tyrosine alkyl ester (DTR) and desaminotyrosyl-tyrosine (DT), and PEG (Figure 1-1). This class of polymers exhibit a high versatility and have been studied for several applications such as drug delivery (Khan, Murthy, & Kohn, 2009; Macri, Sheihet, Singer, Kohn, & Clark, 2012), neural prosthetic devices (D. Lewitus, Smith, Shain, & Kohn, 2011) and bone tissue engineering (Kim, Magno, & Waters, 2012).

In order to fulfill the particular requirements of each application, modifications on the polymer composition have been explored, such as (1) variations on the alkyl pendent chain; (2) changes in percentage of DT; and (3) changes in percentage of PEG. Each variation allows fine tuning of the terpolymers properties.

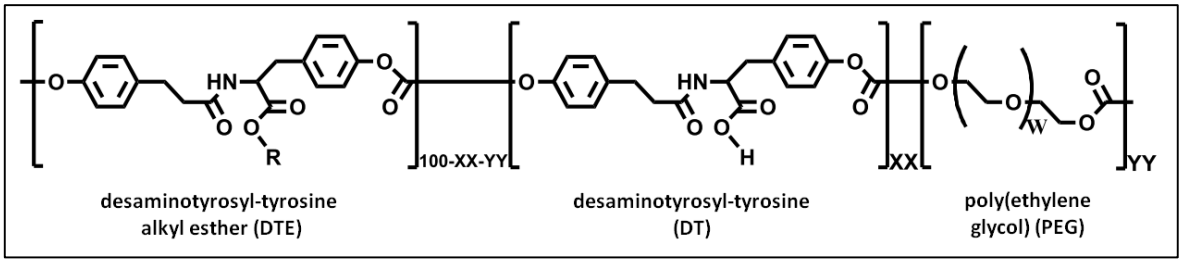

Figure 1-1: Tyrosine derived terpolymers are formed by copolymerization of desamimotyrosyl-tyrosine alkyl ester (DTE), desaminotyrsosyl-tyrosine (DT) and poly(ethylene glycol) (PEG). (Magno, 2012)

While the aliphatic pendent chain increases, the polymer stiffness and T_g are reduced (Varawut Tangpasuthadol, Kohn, Shefer, & Hooper, 1996). Also, the polymer becomes more hydrophobic and, as a consequence, degradation rate is diminished (V Tangpasuthadol, Pendharkar, & Kohn, 2000). It has been observed that larger alkyl pendent chain are associated with better host cell integration, in particular it has been reported a greater osteogenicity (James, Levene, Parsons, & Kohn, 1999).

As reviewed in Magno (Magno, 2012), DT addition accelerates the degradation rate of the polymers by increasing acidity of the molecule and by rising the hydrophilicity of the polymer matrix. DT molecules also increase the erosion rate, because they are capable to solubilize in water and leave the polymer matrix, which lead to mass loss. DT is a stiff

monomer and its incorporation means increased stiffness and higher T_g. However, it has been suggested that this higher stiffness could reduce the water permeability.

The presence of PEG in a terpolymer plays a key role controlling degradation rate and bulk erosion. PEG is a highly hydrophilic and, when the polymer is immersed in an aqueous medium, water tends to associate with PEG segments. The increasing availability of water in the polymer backbone, allow a faster degradation of carbonate bonds near to PEG blocks. However, the carbonate cleavage usually leads to non-soluble remaining molecules and therefore little erosion is observed. PEG is also related with less mechanical strength and lower T_g. An important effect of the incorporation of this monomer is the decrease in protein adsorption and consequently a less integration with host cells. Remarkably, it has been reported that the presence of DT and PEG accelerate the degradation and erosion process of the polymer more than the presence of each monomer alone (D. Y. Lewitus, Rios, Rojas, & Kohn, 2013).

Terpolymers degradation occurs via hydrolysis. The degradation process could be described with a first order kinetic equation since autocatalysis is not expected (V Tangpasuthadol, Pendharkar, Peterson, & Kohn, 2000). DTR have three bonds susceptible to hydrolysis: the carbonate and amide in the backbone, and the ester in the pendent chain (V Tangpasuthadol, Pendharkar, & Kohn, 2000). Generally, hydrolysis first takes place in the backbone carbonate bond, which leads to loss of molecular weight (degradation) but not to mass loss, likely because the degradation products are not soluble enough. For complete erosion happens ester hydrolysis must occur, transforming the DTR into DT, which is more soluble. Enzyme-mediated degradation of terpolymers is unlikely due to the inherent stiffness of the polymer.

The first step to hydrolysis that takes place is water uptake, therefore the permeability of water into the polymer matrix is a determining step in the degradation rate. Due to the hydrophilic nature of DT and PEG, carbonates bonds closer to that monomers will degrade first. This means there will be a preferential cleavage of DTE-PEG or DTE-DT bonds instead of DTE-DTE bonds.

Magno et al. (Magno, 2012) proposed the use of poly(DTE-*co*-10%DT-*co*-01%PEG carbonate) for bone regeneration. Poly(DTE carbonate) was selected due to its osteoconductive properties and its superior strength in comparison with other degradable polymers tried for orthopedics applications such as poly(ε-caprolactone) and poly(ortho ester)s. To overcome the mismatch between polymer degradation and new bone formation, DT and PEG were incorporated in an optimum percentage.

To the best of our knowledge, very little is known about the behavior of tyrosine-derived polycarbonates and its copolymers and terpolymes in an infected environment. In order to know if cell activity affects these polymers, Hooper et al. (Hooper et al., 1998) compared the degradation rate of poly(DTE carbonate) extruded pins *in vivo* and *in vitro*, finding that degradation occurs at comparable rates in both media. Since then, it has been accepted by Hooper et al. as by other authors that there are no enzymatic contributions to poly(DTE carbonate) degradation *in vivo* (Sommerfeld, Zhang, Costache, Vega, & Kohn, 2014). However, it has not been directly studied the effect of bacterial challenge on tyrosine-derived polycarbonate terpolymers.

Implant infection is an actual concern in the biomaterial field. Studying polymer-bacteria interactions allows to better predict the behavior of a polymer in case of bacterial challenge inside the patient's body. In case of tyrosine-derived tepolymers very little is known about the effects of bacteria in polymer properties, such as degradation rate. Also unknown is the ability of bacteria to proliferate on the polymer surface. Studying these phenomena could help move these promising terpolymers from research laboratories to clinical applications.

1.4 Hypothesis and Objectives

In the present study we proposed that bacterial contamination affects the degradation rate of tyrosine-derived polycarbonate terpolymer films *in vitro*. Additionally, the absence of acidic degradation products in terpolymers allows bacteria growth on the culture media and the adhesion over the polymer surface.

The aim of the present study was to assess the effect of bacterial contamination in the *in vitro* degradation of two tyrosine derived polycarbonates, E0000 and E1001(1K) and two poly(hydroxy acids), P(DL)LA and PLGA 50:50. Bacteria evaluated were environmental *Pseudomona* sp., methicillin resistant *S. aureus* and *P. aeruginosa*.

The specific objectives of this work were:

(i) To study the effect in degradation rate of E0000, E1001(1K), P(DL)LA and PLGA 50:50 of spontaneous contamination with an environmental *Pseudomona* sp. occurred in two different culture media - a low nutrient medium (PBS) and an enriched medium (PBS + fibrinogen).

(ii) To study the effect in the degradation rate of E0000, E1001(1K) and P(DL)LA of inoculated bacterial strains - methicillin resistant *S. aureus*, *P. aeruginosa*, and the environmental *Pseudomona* sp. - in a low nutrient culture medium (PBS).

(iii) To study the proliferation, growth and biofilm formation of these bacteria in the presence of each polymer.

This research seeks to expand our knowledge of the interaction between tyrosine-derived polycarbonates and bacteria, analyzing the changes in degradation rate trigger by bacterial presence and evaluating the capacity of this polymers to support bacterial growth and colonization. These findings will help to better understand the behavior of promising biomaterials in two relevant situations: (i) contamination of devices before implantation and (ii) contamination after implantation inside the patient's body.

1.1 Organization of the Document

The present document is divided as follows. Section 2 describes the polymers and bacteria used in this study and the techniques applied to characterize polymer and bacterial behavior. It is also explained the experimental design divided in three parts: (i) study of the effect of spontaneous contamination in polymer degradation rate, (ii) study of effect of clinically relevant bacteria in polymer degradation rate, and (iii) analysis of the biofilm formation of the bacteria in study on polymer surfaces. Afterwards, in Section 3 the results of each part of the experimental design are presented and discussed, including the degradation rate curves, the bacterial growth and images of the biofilms formed. Finally, in Section 4 some relevant conclusions are highlighted. In the annexes is presented a data mining analysis of a library of polyarylates performed in addition to the main research.

2. MATERIALS AND METHODS

2.1 Polymers in study

Tyrosine-derived polycarbonates terpolymers were synthetized by New Jersey Center for Biomaterials as previously reported (Magno et al., 2010). To simplify naming, tyrosine-derived terpolymers of the form poly(DTR-co-XX%-DT-co-YY%-PEG_{Mw} carbonate) are referred as RXXYY(Mw). For example, poly(DTE-co-10%-DT-co-01%-PEG1K carbonate) corresponds to E1001(1K). This study includes two different tyrosine-derived terpolymers: E0000 and E1001(1K). Also two commonly used polymers in biomedical applications, poly(DL-lactic acid) (P(DL)LA) (Aldrich, 531162) and poly(lactic-co-glycolic acid) (PLGA) 50:50 (Sigma, P2191), are included.

2.2 Experimental design

Two scenarios were evaluated to assess the influence of bacteria on the degradation of the polymers studied. First, it was evaluated the effect of spontaneous contamination with environmental *Pseudomona* sp. of samples incubated at 37°C. Second, the effect of controlled contamination on the media was evaluated for two clinically relevant bacteria, *P. aeruginosa* and methicillin resistant *S. aureus,* plus environmental *Pseudomona* sp. Later, the adhesion of bacteria on polymer surfaces and possible biofilm formation was assessed by microscopic observation. Analytical details associated with polymer processing and characterization, and microbiological analysis are explained later in the following sections.

2.2.1 Polymer-bacteria interactions in medium contaminated with environmental *Pseudomona* sp.

Compression molded film disks (6.35 cm diameter, 100-150 µm) were obtained from E0000, E1001(1K), P(DL)LA and PLGA 50:50. Previous to incubation, molecular weight (M_w) and water content of the films were assessed. The samples were incubated for different times in 10 mL of two different media under non sterile conditions: (i) phosphate buffered saline (PBS) (BM-1340, Winkler) and (ii) fibrinogen from bovine plasma (Sigma, F8630), diluted in PBS with a concentration of 3 mg/mL (Figure 2-1). Each film disk was incubated

separately in scintillation vials with 5 mL of the respective solution and removed at selected time points chosen in a way that encompass the half degradation time of each polymer (Table 2-2). Three repetitions were performed for each incubation time. Fibrinogen solution was replaced weekly while PBS remained constant. The pH of media was measured in order to confirm the absence of changes. After incubation, each sample was superficially dried with Kimwipes® and stored at -20°C for further M_w or water uptake analysis.

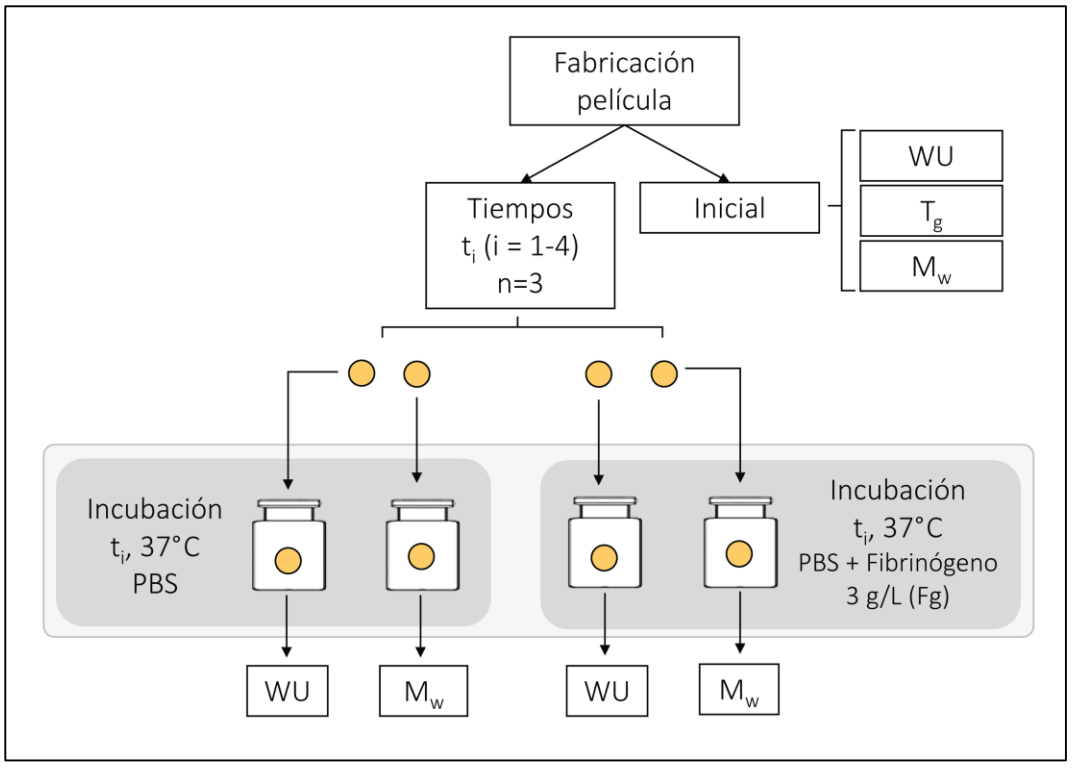

Figure 2-1: Scheme of the methodology for assessing the effect of spontaneous contamination with environmental *Pseudomona* sp. Polymer disks of E0000, E1001(1K) and P(DL)LA were obtained from different compression molded films, and incubated in (i) PBS or (ii) fibrinogen diluted in PBS (3 g/L) for further molecular weight (M_w) and water uptake (WU) determination.

Table 2-1: Selected time points "i" for the 4 polymers in study.

Polymer	i=0	i=1	i =2	i=3	i=4
E0000	0	5 d	15 d	35 d	105 d
E1001(1K)	0	1 d	6 d	18 d	55 d
PDLLA	0	1 d	6 d	18 d	55 d
PLGA 50:50	0	5 h	1 d	5 d	15 d

2.2.2 Polymer-bacteria interactions in medium contaminated with clinically relevant bacteria

E0000, E1001(1K) and P(DL)LA samples (6.35 cm diameter, 100-150 µm) were incubated at 37°C under normal atmospheric conditions (aerobic environment) in 10 mL of sterile PBS inoculated with approximately 10^5 CFU/mL of *Pseudomona aeruginosa*, methicillin resistance *Staphylococcus aureus*, or environmental *Pseudomona* sp. (Figure 2-2). Each sample was incubated separately in a sterile centrifuge tube and removed at 15 or 30 days of incubation for M_w analysis. Additionally, bacterial concentration was measured at days 7, 15, 22 by aerobic plate count in plate count agar (PCA).

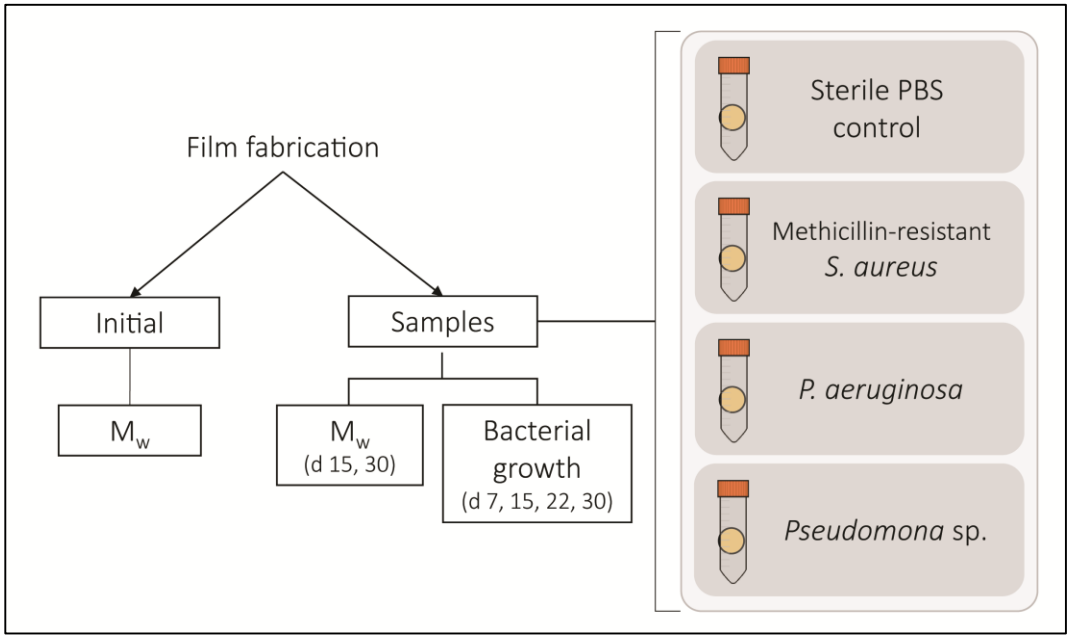

Figure 2-2: Scheme of the methodology for assessing the effect of clinically relevant bacteria. Polymer disks of E0000, E1001(1K) and P(DL)LA were obtained from compression molded films and incubated in (i) sterile PBS or with 10^5 CFU/mL of (ii) methicillin-resistant *S. aureus*, (iii) *P. aeruginosa* and (iv) environmental *Pseudomona* sp. M_w and bacterial growth was assessed at several incubation times.

2.2.3 Bacterial adhesion

To observe the bacterial adhesion on polymer surfaces and possible biofilm formation, samples of E0000 (6.35 cm diameter, 100-150 μm) were incubated at 37°C in 10 mL of the following incubation media:

(i) Sterile PBS (control)
(ii) Low concentration of *P. aeruginosa* (approx. 10^5 CFU/mL)
(iii) High concentration of *P. aeruginosa* (approx. 10^8 CFU/mL)
(iv) Low concentration of environmental *Pseudomona* sp. (approx. 10^5 CFU/mL)
(v) High concentration of environmental *Pseudomona* sp. (approx. 10^8 CFU/mL)

After 10 days of incubation the samples were removed and observed under microscope.

2.3 Polymer analysis

2.3.1 Polymer processing

Polymer films were fabricated by compression molding, using a hydraulic press (Dake Corp, Model 44-225 Lab Press). Approximately 200 mg of polymer were placed between the plates of a steel mold covered with Kapton® film, and spaced with steel shims of 100 μm. The mold was located between the platens of the hydraulic press, which was pre-heated at process temperature (T_p), and maintain with no pressure for 5 minutes to equilibrate temperature. The pressure was raised to 15 metric tons. After one minute, the pressure was released slowly and the mold with the film was cooled for 15 minutes. Polymer films were removed and storage at -20°C for further analysis. The T_p was adjusted to each polymer ranging between 35-75°C above glass transition temperature (T_g) (Table 2-2) based on previous experiments performed by Magno et al. (Magno et al., 2010). The T_g was reported by Kohn's research group for E0000 and E1001(1K) and it was measured by differential scanning calorimetry (DSC) for PLGA 50:50 and P(DL)LA.

For DSC analysis, a sample of approximately 5 mg was placed in DSC equipment (Metter Toledo, model 821e) inside a sealed aluminum pan, and heated from 0° C to 150° C,

increasing temperature at a rate of 10° C per minute. The sample was kept in 150° C for 2 minutes, and cooled again to 0°C. After 2 minutes at this temperature, a second heat cycle was performed. The T_g of each polymer was determined as the midpoint of the transition from the second heat cycle.

Table 2-2: Processing temperature (T_p) was defined for each polymer by successive trials, ranging from 35 to 75°C above the glass transition temperature (T_g).

	T_g (°C)	T_p (°C)
E0000	93.3	158
E1001(1K)	95.3	165
PLGA 50:50	42.9 *± 2.7*	82
P(DL)LA	46.3 *± 2.2*	91

2.3.2 Polymer degradation

Polymer degradation was characterized measuring M_w of the samples before processing, and before and after incubation. Degradation was defined as M_w remaining percentage ($\%M_w/M_{w0}$), defined as the percentage of M_w at each time point in relation to the initial M_w of the film (M_{w0}) showed in Table 2-3. M_w was obtained using size exclusion chromatography (SEC) (columns MIXED-C PLgel 5 µ, pump Knauer model 14163, detector Technology Corporation model Dawn EOS) relative to commercially polystyrene standards. The samples were dissolved in 500 µL of tetrahydrofuran (THF) (LiChrosolv 1.08101, Merck). The solvent flow was settled to 1 mL/min and the pressure of the pump was 11-15. Data obtained was smoothed adjusting a Gaussian model to each curve in OriginLab 8 Software.

Table 2-3: Initial M_w is represented as mean ± *SD* of three samples.

Polymer	M_{w0} (kDa)
E0000	172.14 *± 14.4*
E1001(1K)	96.15 *± 8.41*
PLGA 50:50	64.70 *± 8.12*
P(DL)LA	143.63 *± 7.4*

2.3.3 Polymer water uptake

Water uptake (WU) was obtained by thermogravimetric analysis (TGA) (Mettler Toledo TGA/SDTA 851e), heating the sample from 30°C to 200°C at a rate of 5°C per minute. The water content was obtained using the following equation

$$WU\% = 100 \cdot \frac{M_{loss}}{M_{dry}} - WU_0 \quad (2\text{-}1)$$

Where M_{loss} is the change in the mass of the sample between 30°C y 200°C, and M_{dry} is the mass of the sample at 200°C. WU_0 is the percent of water content of the film before incubation.

2.4 Microbiological analysis

2.4.1 Bacterial identification

For 16S rDNA cloning experiments, E0000 films were incubated in non-sterile PBS and fibrinogen solution. After one week, 100 µL of both samples were diluted (10^{-3}) and plated in PCA (247940, Difco). Three isolated bacterial colonies were added to 10 mL TSB and incubated for 24 h at 37°C in order to increase bacterial concentration. After this, bacterial cells were centrifuged for 10 min at 5000 × g. DNA was extracted using gram negative bacteria protocol of GeneJet Genomic DNA Purification Kit (Thermo Scientific, #K0721, #K0722) and then stored at -20°C. Polymerase chain reaction (PCR) was done using approximately 2 ng of DNA. For PCR, 16S rDNA bacteria primers 1492R (5'-TACGGYTACCTTGTTACGACTT-3') and 27F (5'-AGAGTTTGATCMTGGCTCAG-3') were used. The amplification was performed for 25 cycles (94°C for 1 minutes, 57°C for 2 minutes and 72°C for 2 minutes per cycle). DNA was sent to Macrogen (Macrogen Inc., Rep. of Korea) for sequencing. Sequence analysis was performed using NCBI BLASTN algorithm (www.ncbi.nlm.nih.gov).

2.4.1 Bacterial growth

The aerobic mesophilic bacteria growth was measured by spread-plate technique using a PCA growth medium (247940, Difco). First, 100 µL of the samples were diluted depending on the expected bacteria concentration, and then placed and circularly spread in culture plates under sterile conditions. They were incubated for 24 h at 37°C and the colonies formed were manually count. Each bacterial growth assay was performed in triplicate.

2.4.2 SEM

The possible biofilm formation over polymer surface was observed using scanning electron microscopy (SEM). Samples of E0000 were incubated in *P. aeruginosa* in a concentration of 10^5 UFC/mL and environmental contamination bacteria in concentrations of 10^5 and 10^8 UFC/mL. After 10 days of incubations the samples were removed and fixed using glutaraldehyde 2.5%. Then the samples were dried by critical point drying and coated in gold using a sputter coater for further observation using SEM.

2.5 Statistical analysis

2.5.1 Error propagation

Degradation rate was obtained as the percentage of M_w at each time point in relation to the initial M_w of the film. Therefore, measurement errors in initial M_w were propagated to each time point.

Considering the function

$$f = 100\ \frac{M_w}{M_{w0}} \tag{2-2}$$

Being M_w and M_{w0} uncorrelated variables, the error of the function (Lee & Forthofer, 2006) was defined as

$$\sigma_f = \sqrt{\left(100\frac{M_w}{M_{w0}}\right)^2\left[\left(\frac{\sigma_{M_w}}{M_w}\right)^2 + \left(\frac{\sigma_{M_{w_0}}}{M_{w_0}}\right)^2\right]} \tag{2-3}$$

2.5.2 Statistical analysis

Statistical significance of the differences of the means for samples incubated PBS and fibrinogen solution treatments was assessed by a two sample t-test for each time point calculated using Excel®. To assess statistical significance of the differences of the means for samples incubated in contaminated media and control, one-way ANOVA was performed using R software. In both cases, the calculations were performed considering error propagation. A difference was considered significant if *p*-value was less than the significance level 0.05.

3. RESULTS AND DISCUSSION

3.1 Polymer-bacteria interactions in media contaminated with environmental *Pseudomona* sp.

3.1.1 Bacteria identification

Preliminary, the contamination of the medium was corroborated by aerobic plate count of the culture media of PLGA 50:50 and E0000 samples after 2 weeks of incubation. For both polymers incubated either in PBS or fibrinogen solution, it was observed a bacterial concentration higher than $2x10^7$ CFU/mL.

Accordingly, non-sterile PBS and fibrinogen solution media containing E0000 samples incubated for one week showed bacterial growth for both cases. Interestingly, the concentration of bacteria present in medium containing fibrinogen was considerably higher ($> 2x10^7$ CFU/mL) than the concentration of bacteria in medium containing only PBS ($2x10^5$ CFU/mL). The homogenous aspect of the colonies indicated that a single kind of microorganism colonized the samples, most likely a bacteria (Figure 3-1). It cannot be discarded that other species were also present in the culture medium, but in small quantities.

Additionally, PBS and fibrinogen solution alone without polymer film were also incubated under non-sterile conditions. It was observed that bacteria also proliferates in both cases, concluding that the presence of the polymer film was not crucial for the proliferation of bacterial contamination.

Several procedures were carried out in order to identify the bacterial strain. First, it was tested its capability to growth in MacConkey agar, with a positive result (Figure 3-2). The proliferation of the bacteria in this substrate implies the Gram negative nature of this microorganism. The Gram staining corroborated this previous result (Figure 3-3). The observation under optical microscope also showed a rod-shaped form with tendency to form clusters with chain shape.

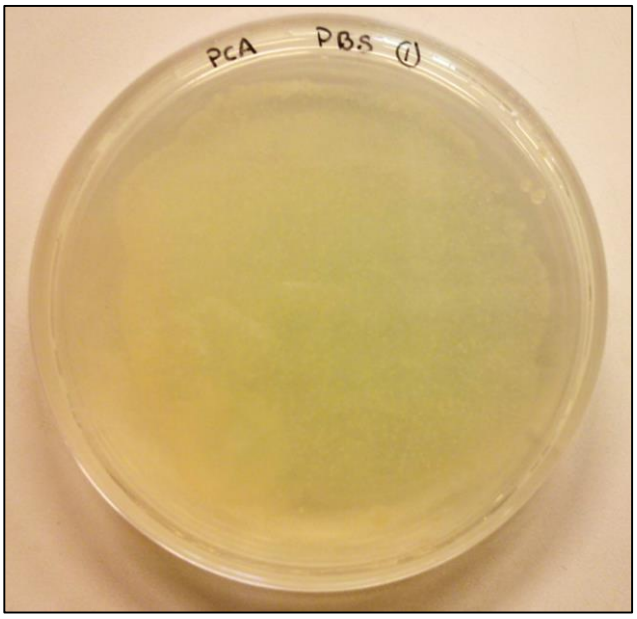

Figure 3-1: Example of plate count in PCA of bacteria involved in samples contamination. Homogeneity of the colonies indicates the presence of mainly one bacterial species.

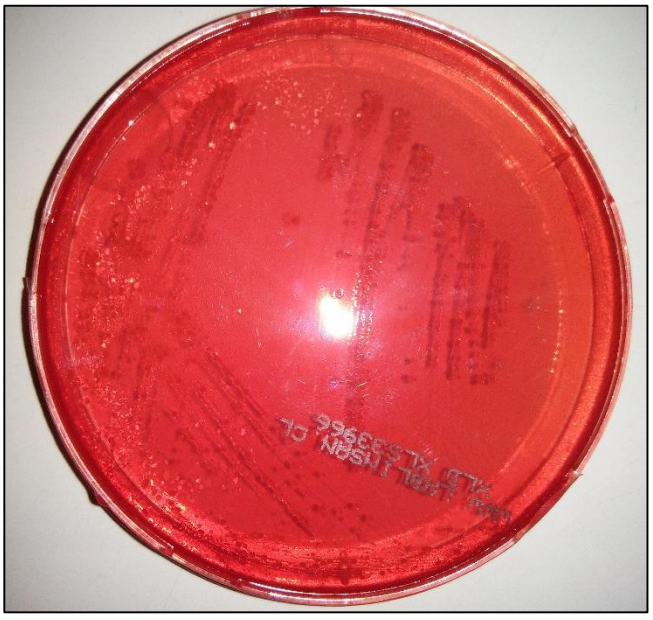

Figure 3-2: Bacteria involved in samples contamination grew in Agar MacConkey, indicating gram negative nature.

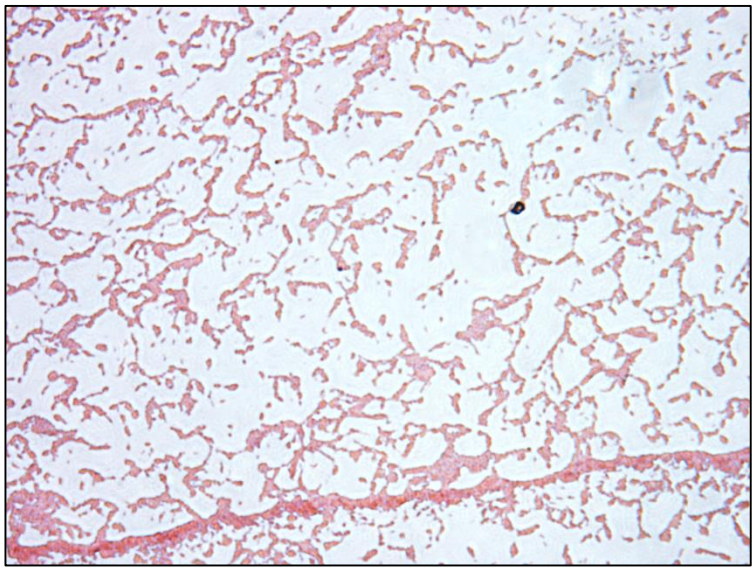

Figure 3-3: Bacteria involved in samples contamination was gram-negative and rod-shaped. Observation under optical microscope at x40.

A molecular analysis was performed to identify the bacterial species. 16S rDNA sequence was contrasted with NCBI database through BLAST (results are showed in detail in Annex A). They were obtained hits with coverage and identity to the query of 99%. All better matches indicated that the environmental bacteria involved in sample contamination was a *Pseudomona* sp. The better match was obtained was *Pseudomona indoloxydans* strain, a recently reported specie isolated from a pesticide-contaminated site (Manickam, Ghosh, Jain, & Mayilraj, 2008). However, this specie have a high similarity (99%) with *Pseudomona pseudoalcaligenes*, which also match with high coverage and identity. In order to identify with precision the specie and strain of this bacteria, future research should include 16S rDNA sequencing with specific *Pseudomona* primers.

From molecular analysis is known that bacterial contamination was certainly generated by a *Pseudomona* species. The spontaneous contamination and proliferation with a *Pseudomona sp*. was not surprising, due to its high environmental abundance. It is not discarded the presence of other microorganisms, but in little quantities. The lack of important amount of

other bacteria may be due to the high nutrient scarcity of the experimental conditions in which only a small group of microorganisms could proliferate.

3.1.2 Molecular weight retention

The degradation profile of E0000 was obtained up to 100 days of incubation in both media (Figure 3-4). Degradation of samples incubated in PBS was significantly lower than samples incubated in fibrinogen solution, after 15 days of incubation (p-values < 0.01). Half degradation time of films incubated in PBS was approximately 45 days of incubation, while for the films incubated in fibrinogen solution it was around 20 days. The faster degradation is associated with faster erosion, which is observed because samples incubated in fibrinogen solution were more fragile than the ones incubated in PBS after 100 days.

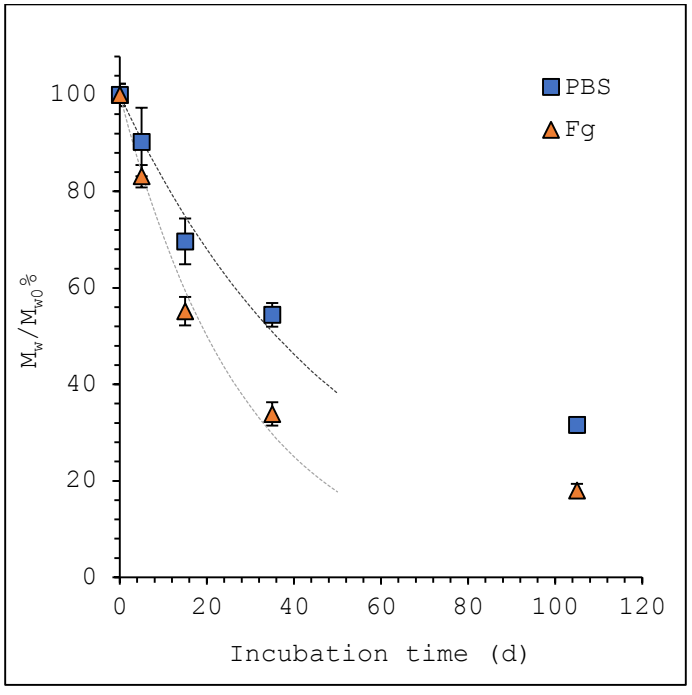

Figure 3-4: E0000 disks incubated in PBS (blue squares) presents a lower degradation rate than E0000 disks incubated in fibrinogen solution (orange triangles). Points are presented as mean ± SD of three samples, considering the propagation of error of initial M_w.

A first order kinetic equation of the form was used to fit the data for the first 40 days of incubation:

$$\frac{M_w}{M_{w_0}}\% = 100\, e^{-kt} \quad (3\text{-}1)$$

where k is a parameter and t the time. Polymer disks incubated in PBS presented a smaller degradation constant ($k = 8 \times 10^{-4}\ h^{-1}, R^2 = 0.95$) than polymer films incubated in fibrinogen ($k = 1.4 \times 10^{-3}\ h^{-1}, R^2 = 0.98$).

E1001(1K) shows a similar behavior than E0000 (Figure 3-5). However, the variability of the measurements was higher, probably due to lower initial M_w measured (45 kDa). Samples incubated in PBS have a significantly slower degradation rate in relation with samples incubated in fibrinogen solution after 26 days of incubation (p-values < 0.03), with half degradation times of approximately 80 (extrapolated) and 35 days, respectively. A first order kinetic equation fits the data for both cases during all the incubation time. As in E0000, polymer disks incubated in PBS presented a smaller degradation rate ($k = 6 \times 10^{-3}\ h^{-1}, R^2 = 0.94$) than polymer films incubated in fibrinogen ($k = 1.8 \times 10^{-2}\ h^{-1}, R^2 = 0.90$).

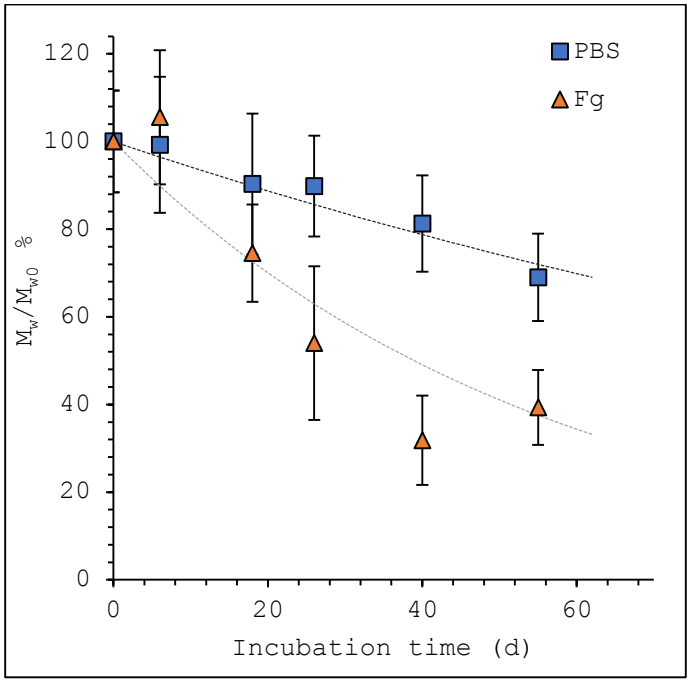

Figure 3-5: E1001(1K) disks incubated in PBS (blue squares) degrade slower than E1001(1K) films incubated in fibrinogen solution (orange triangles). Points are presented as mean ± SD of three samples, considering the propagation of error of initial M_w.

PLGA 50:50 degradation profile was obtained over two weeks of incubation (Figure 3-6). Degradation rate of PLGA incubated in PBS was not significantly different from samples incubated in fibrinogen solution (*p*-values > 0.16). Half degradation times obtained for samples incubated in PBS and fibrinogen solution were 11.3 days and 10.4 days, respectively. A first order kinetic equation is well suited to describe the data of PLGA. Degradation rate parameters were similar for samples incubated in PBS ($k = 6{,}1 \times 10^{-2}\ h^{-1},\ R^2 = 0.99$) and those incubated in fibrinogen solution ($k = 6.7 \times 10^{-2}\ h^{-1}, R^2 = 0.96$).

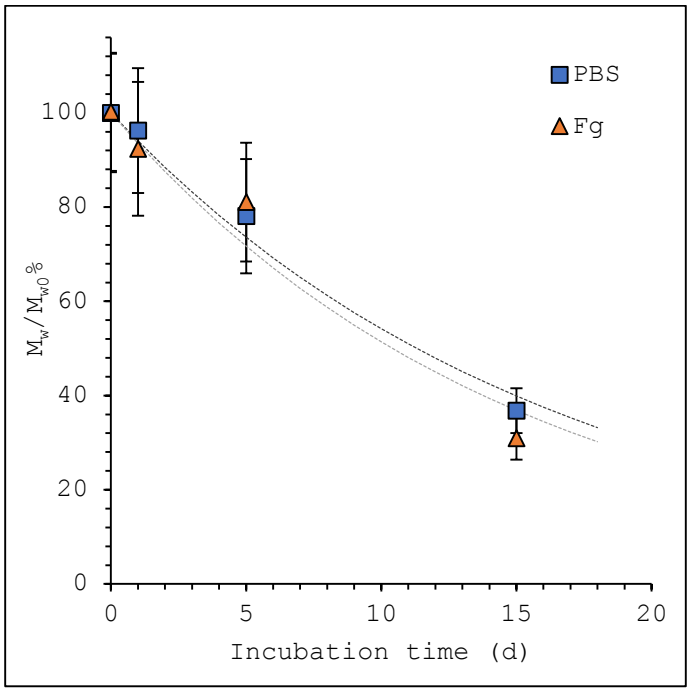

Figure 3-6: PLGA 50:50 samples incubated in PBS (blue squares) has no significantly different degradation rate than samples incubated in fibrinogen solution (orange triangles). Points are presented as mean ± SD of three samples, considering the propagation of error of initial M_w.

P(DL)LA degradation profile was also obtained for 55 days of incubation (Figure 3-7). Polymers incubated in PBS seem to degrade faster in PBS than the ones incubated in fibrinogen, however the difference was not significant (*p*-values > 0.08). Half degradation times of both films were after 55 days of incubation (i.e., out of the time span studied). A

first order kinetic equation was used to fit the data, obtaining a slightly higher degradation rate for films incubated in PBS ($k = 2.5 \times 10^{-2}\ h^{-1}, R^2 = 0.84$) than films incubated in fibrinogen solution ($k = 1.6 \times 10^{-2}\ h^{-1}, R^2 = 0.39$). However, it was observed that this kinetic equation did not capture the tendency of the data as well as in the previous cases.

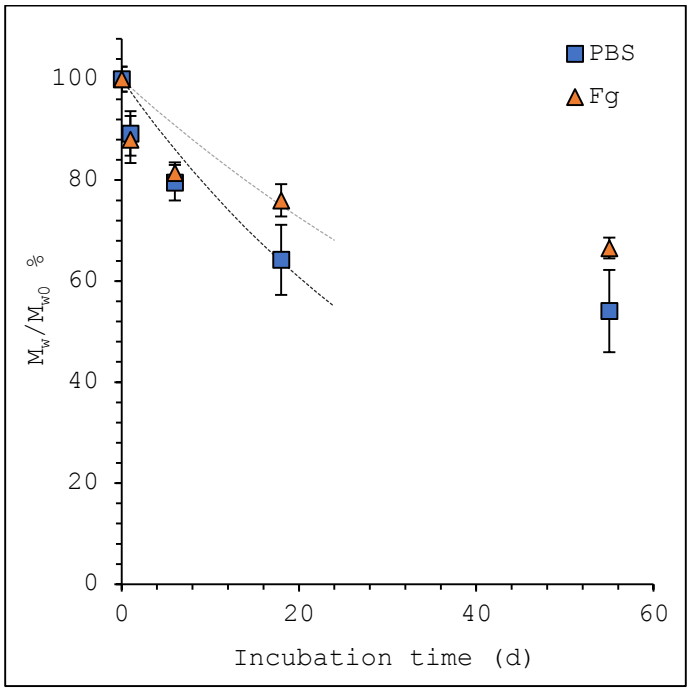

Figure 3-7: P(DL)LA samples incubated in PBS (squares) has a slightly lower degradation rate than samples incubated in fibrinogen solution (triangles). Points are presented as mean ± SD of three samples, considering the propagation of error of M_{wi}.

It has been described that poly(hydroxyl acids) have acidic degradation byproducts that exert an autocatalytic effect. In these experiments PBS media remained constant during all degradation period, which could generate a decrease in local pH. However, it was observed that pH of media with PLGA samples ranged between 7.5 and 8.5 after 2 weeks of incubation. These results agree with previously reported data that showed that pH of incubation media of PLGA decrease after more than 20 days of incubation (Farahani, Entezami, Mobedi, & Abtahi, 2005; Oh et al., 2006). In the case of media with P(DL)LA samples, pH was 6.38 after 2 weeks of incubation at 45°C. Previous studies have also showed that PLA degradation did not seem to alter pH of the medium until 40 weeks of incubation

(Hofmann et al., 1990; Mainil-varlet et al., 1997). However, for P(DL)LA it has been reported a notorious decrease in pH after 2 week of incubation (Mainil-varlet et al., 1997).

The first order kinetic equation model used to describe the degradation rate of E0000, E1001(1K) and PLGA 50:50 for the first 40 days of incubation is widely used in literature for modeling degradation profiles of polyesters such as PLA and PLGA (Farahani et al., 2005; Kenley, Lee, Mahoney, & Sanders, 1987; Lyu et al., 2007) and tyrosine derived polycarbonates (Ertel & Kohn, 1994; Magno, 2012; V Tangpasuthadol, Pendharkar, & Kohn, 2000). However, it was observed that while degradation increases, this first order kinetic equation is not suited to describe the data anymore. This is consistent with previous studies that proposed the need of more than one model to fit de degradation data in poly(hydroxy acids) (Lyu et al., 2007).

The faster degradation of E0000 and E1001(1K) samples in fibrinogen solution in comparison with non-sterile PBS was not triggered only by the presence of fibrinogen. Our collaborators from Kohn's research group studied the possible effect of fibrinogen in degradation rate of tyrosine-derived polycarbonates. They measured the M_w remaining after 5 weeks of incubation at 37°C in four sterile media: (i) PBS alone, (ii) PBS with the antibacterial agent sodium azide (NaN_3), (iii) PBS with fibrinogen, and (iv) PBS with fibrinogen and NaN_3 (Figure 3-8). In this experiment they observed that the presence of fibrinogen itself did not alter the degradation rate of E0000 in comparison with PBS alone. They also added bactericidal agent Sodium Azide (NaN_3) to prevent possible media contamination, observing that samples tend to degrade faster in the presence of the bactericidal agent.

Considering that the presence of fibrinogen in culture media under sterile conditions has no effect by itself in degradation rate of these terpolymers, the main factor accelerating the degradation process must be the higher amount of bacteria in the media and its enzymatic activity. However, there are no previous evidence that polycarbonates could undergo enzymatic degradation. Moreover, it has been reported that these polycarbonates are too stiff and lack a flexible backbone to interact with the active site of enzymes (Magno, 2012;

Sommerfeld et al., 2014), and similar *in vivo* and *in vitro* degradation results had also led to that conclusion (Hooper et al., 1998).

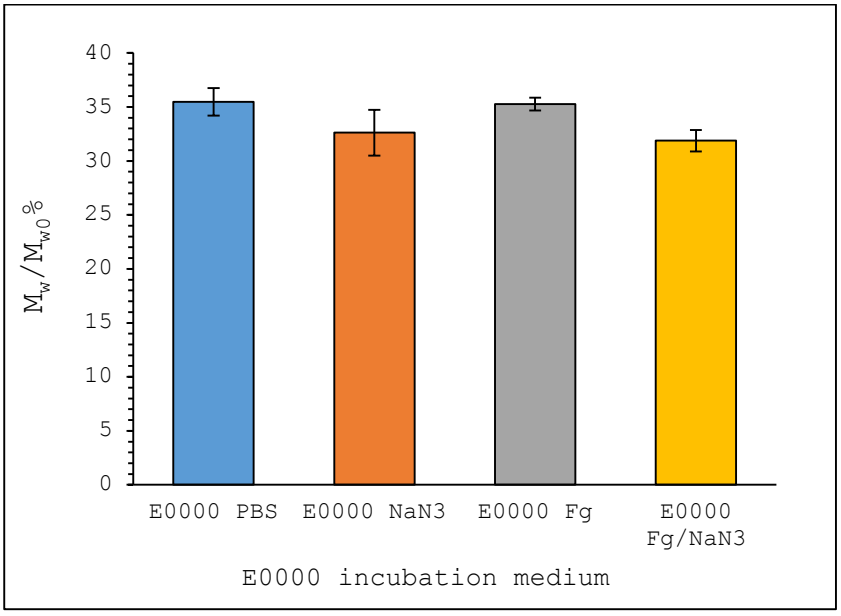

Figure 3-8: Degradation of E0000 in sterile conditions is not be affected by fibrinogen presence in culture media, but bactericidal agent, sodium azide (NaN_3), may accelerate the degradation rate of E0000. Points are presented as mean ± SD of two samples.

In the other hand, P(DL)LA and PLGA 50:50 did not exhibit an accelerated degradation rate at higher amount of environmental *Pseudomona* sp., which may indicate the absence of enzymatic activity over these polymers. This observation agrees with previous results reported by Hoffman et al. (Hofmann et al., 1990), who reported that the presence of clinically relevant bacteria such as *S. aureus* and *S. epidermidis* in culture media did not affect PLA degradation rate. A similar finding was reported by Mainil-Varlet et al. (Mainil-Varlet et al., 2001) who observed that the generation of an infection by *S. aureus* did not affect the *in vivo* degradation rate of P(L)LA and P(L/DL)LA. Additionally, other authors have suggested that these poly(hydroxyl acids) did not undergo enzymatic degradation based on similarities between their *in vivo* and *in vitro* degradation, although William et al. (Williams & Mort, 1977) showed that poly(hydroxyl acids) are susceptible to some

particular enzymes such as pronase, proteinase K and bromelain, which are not produced bu the microorganisms in study.

3.1.3 Water uptake

The water content of E0000 samples incubated in both PBS and fibrinogen solution was obtained at 35 days and 105 d of incubation (Figure 3-9). During the first 35 days the water absorption difference between treatments was negligible (*p*-value = 0.40). At 105 days of incubation, the water content of films incubated only in PBS turns significantly lower than the absorbed water of samples incubated in fibrinogen solution (*p*-value = 0.01).

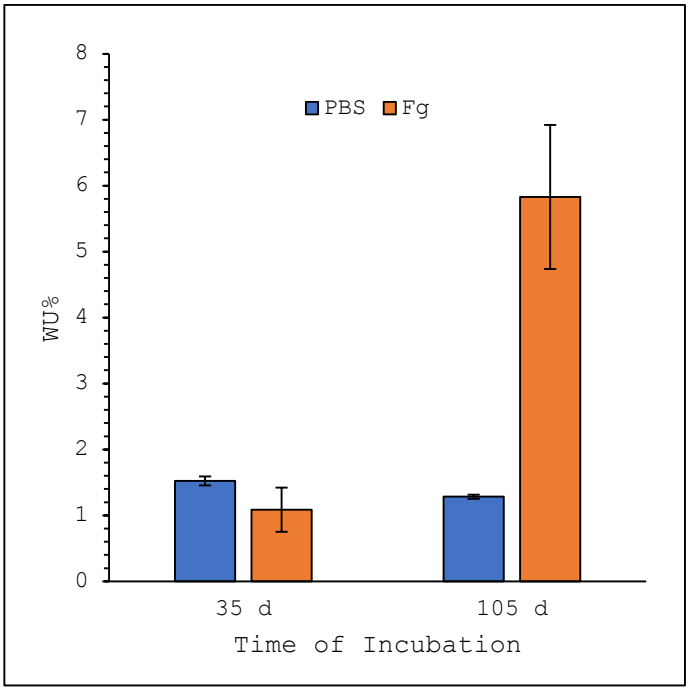

Figure 3-9: E0000 disks incubated in PBS (blue) have a lower water content than E000 disks incubated in fibrinogen solution (orange) in the latest stages of degradation. No significant difference on water uptake is observed in the early stages of incubation. Columns are presented as mean ± SD of three samples.

The water content in E1001(1K) samples were obtained at 18 days and 55 days of incubation (Figure 3-10). It was observed that this polymer is capable to absorb high amounts of water than E0000. The difference between water content between the two treatments was

negligible at 18 days (p-value = 0.93), but at 55 days the films incubated in PBS absorb a significantly lower amount of water than samples incubated in fibrinogen solution (p-value < 0.01).

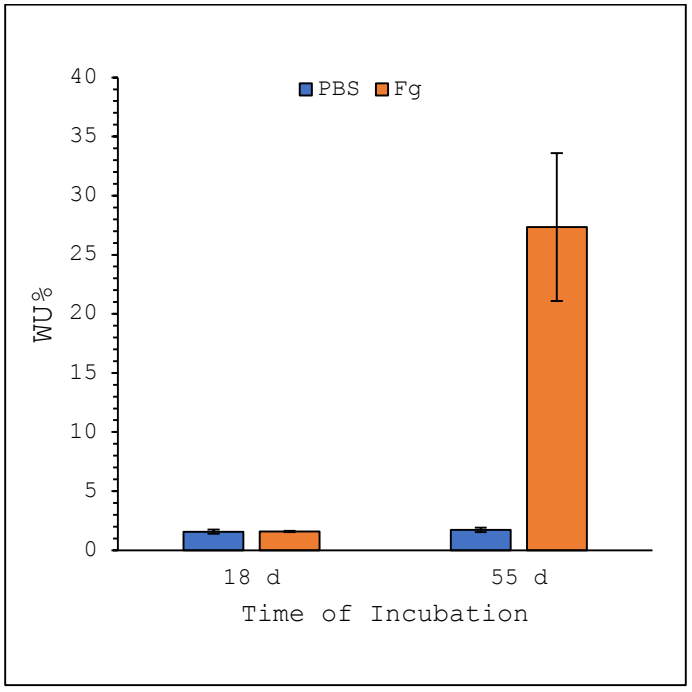

Figure 3-10: E1001 disks incubated in PBS (blue) have a lower water content than E000 disks incubated in fibrinogen solution (orange) in the latest stages of degradation. No significant difference on water uptake is observed in the early stages of incubation. Columns are presented as mean ± SD of three samples

Changes in water uptake were observed only in advances stages of degradation, but noticeable changes in M_w were observed from the first stages of degradation. This result implies that water uptake was not triggering the faster degradation, rather the increase in degradation may triggered the higher water uptake.

3.2 Polymer-bacteria interactions in medium contaminated with clinically relevant bacteria

3.2.1 Physical appearance during degradation

The changes in physical appearance were different depending on the polymer. Through all degradation time E0000 and E1001(1K) remained transparent. It was not observable mass loss in any of them. Moreover, after 30 days degradation period, they were slightly more brittle. On the contrary, P(DL)LA changes its appearance from transparent to opaque white in less than a week of incubation. There was no evidence of mass loss; instead it was observed an increase in sample diameter. P(DL)LA samples were very brittle at the end of incubation period, making difficult to remove them from the medium. Interestingly, polymer samples in sterile environment turned opaque and rubbery before samples incubated in contaminated media.

3.2.2 Molecular weight retention

After 15 days of incubation, there was not found a significance difference in M_w retention of E0000 samples incubated in different culture media (*p*-value = 0.42) (Figure 3-11). After 30 days of incubation samples exhibited a higher degree of degradation than before, but no significance difference was observed either (*p*-value = 0.80).

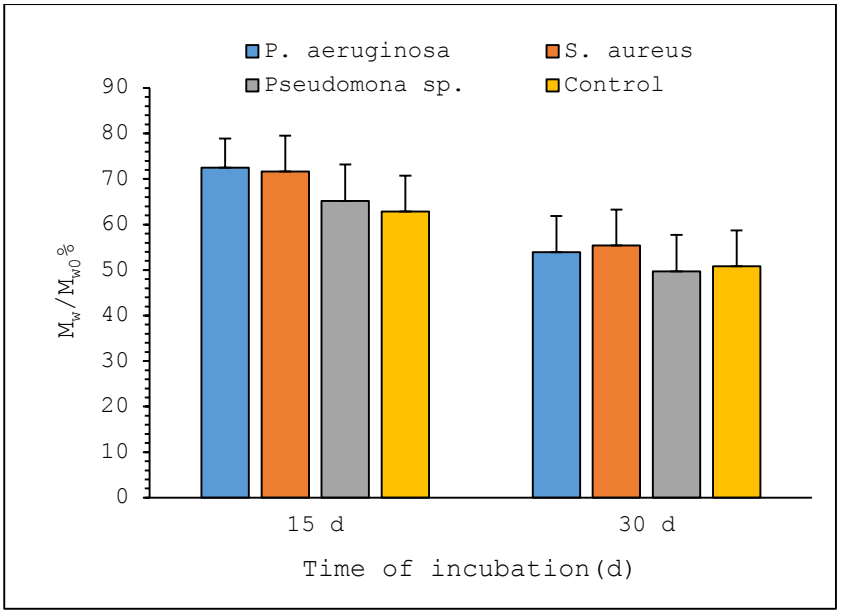

Figure 3-11: No significance difference was observed in E0000 samples between contaminated media or sterile PBS at 15 or 30 days of incubation (*p*-values = 0.42, 0.80). Columns are presented as mean ± SD of three samples.

It was observed that degradation of E0000 samples in controlled contamination media was comparable to degradation of E0000 samples incubated in non-sterile PBS obtained previously (Section 3.1) (Figure 3-12). However, the degradation of samples in fibrinogen solution obtained before was faster, likely due to its high bacterial concentration.

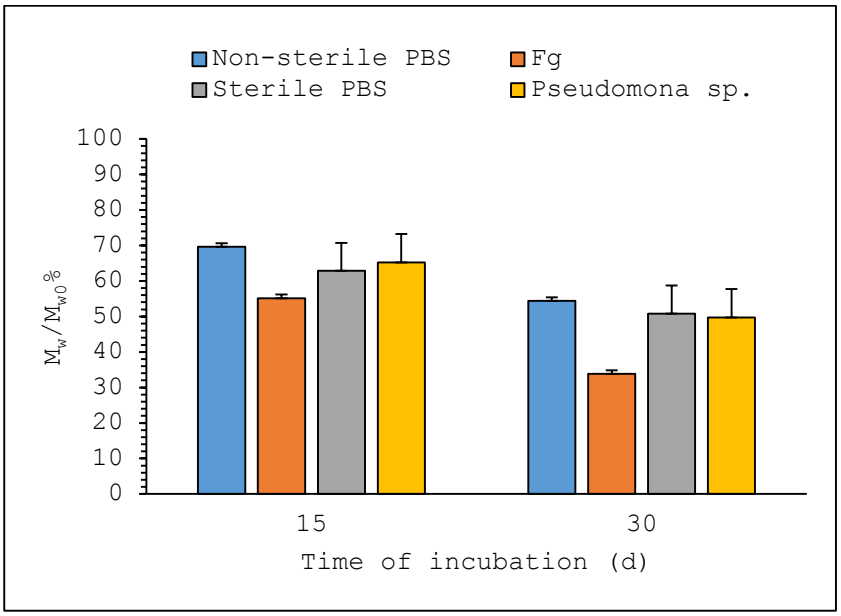

Figure 3-12: Comparison of M_w% remaining of E0000 samples at 15 and 30 days of incubation for spontaneous contamination (non-sterile PBS [blue] and fibrinogen solution [orange] media), and controlled conditions (sterile PBS [grey] and *Pseudomona* sp. [yellow]).

In the case of E1001(1K), no significant difference was observed in M_w retention of samples incubated in different media at 15 or 30 days of incubation (*p*-values = 0.08, 0.39, respectively) (Figure 3-13). Also, it was not observed higher degradation at advanced stages of incubation. Remarkably, the percent of remaining M_w of the samples was smaller than the observed in the previous study, in which the polymer incubated in non-sterile PBS showed a percent of M_w remaining of 91% and 84%, at 15 and 30 days, respectively. In this experiment, E1001(1K) at 15 or 30 days of incubation in sterile PBS presented a remaining M_w ranging from 60% to 45%. The initial M_w was also very different between both experiments, being of 45 kDa in the first experiment and 96 kDa in the second one.

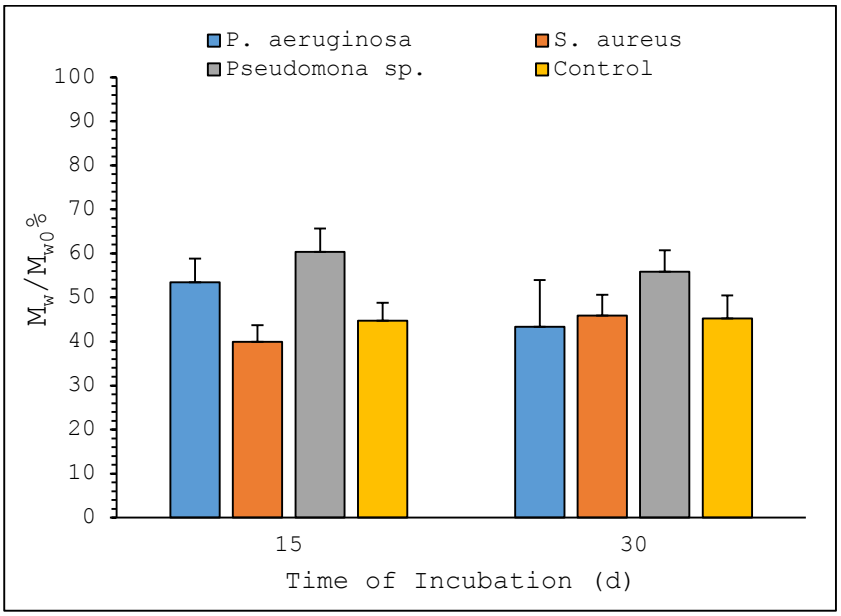

Figure 3-13: E1001(1K) samples did not showed a different degradation rate between different treatments (*p*-values = 0.08, 0.39). No further degradation was observed at late stages of incubation. Columns are presented as mean ± SD of three samples.

P(DL)LA samples showed a different behavior (Figure 3-14). Polymer samples incubated for 15 days showed a significant difference between treatments in the ANOVA test (*p*-value < 0.001). Pairwise comparison of treatments showed that there was significance difference in control samples in relation to the others treatments (*p*-values < 0.005), while differences between contaminated samples were not significant. At 30 days of incubation it was observed that M_w of P(DL)LA samples in sterile PBS was not detectable by the equipment, probably due to being below the detection threshold.

The initial M_w of P(DL)LA samples in the second experiment was equal to the obtained in the first experiment. However, in this second experimental procedure polymer samples degrade faster. In the previous experiment remaining M_w at 30 days of incubation for samples in non-sterile PBS was approximately 61%, while in this experiment it was 25% for samples incubated in medium with environmental *Pseudomona* sp. and undetectable for samples incubated in sterile PBS.

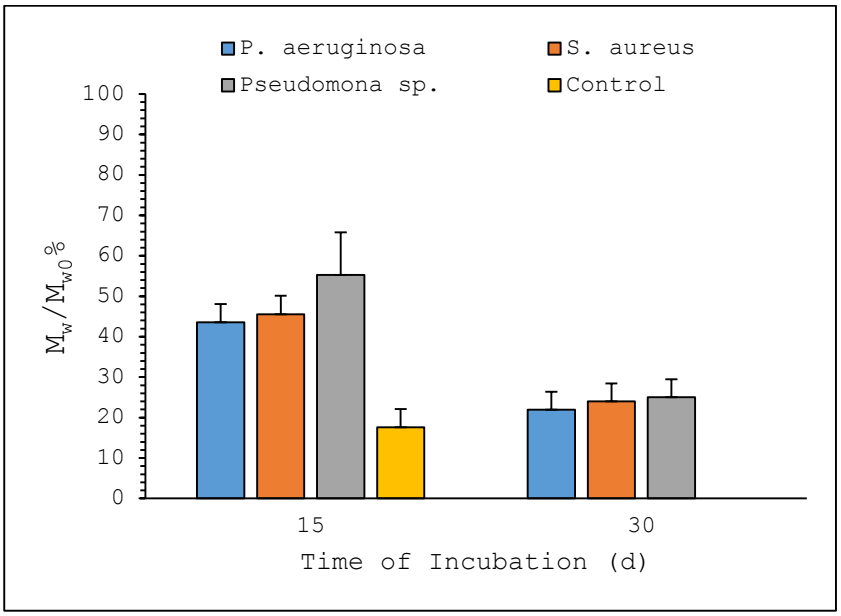

Figure 3-14: There was no difference in P(DL)LA degradation between media contaminated with *P. aeruginosa*, *S. aureus* or environmental *Pseudomona* sp., but samples incubated in sterile PBS showed a faster degradation. M_w after 30 days of incubation could not be obtained because it was below the detection threshold of the equipment. Columns are presented as mean ± SD of three samples.

In these experiments it was expected to replicate the previous results in which bacterial presence triggered a faster degradation of tyrosine-derived polycarbonates. However, in this case there was not found any significant difference between control and treatments in terpolymers samples. Correspondingly, there was not found any difference in degradation rate between samples incubated in different kind of bacteria. It may be hypothesize that bacterial concentration ranging from 10^5-10^6 CFU/mL was too low to affect significantly the degradation process of the polymers, because in the previous study it was observed an effect in degradation when samples spontaneously contaminated had a bacterial concentration higher than 10^7 CFU/mL.

M_w of E0000 samples was the only that have comparable results between the first and second experiment. In the case of E1001(1K) it was observed great differences between initial M_w in the first and second experiment that made very difficult to compare both results. In the second set of experiments the initial M_w was twice of the obtained in the first experiment,

but the degradation rate was faster which is not intuitive considering that high initial M_w samples commonly degrade slower than low M_w samples. Additionally, in the last experiment there was not observed a decrease in M_w between 15 and 30 days of degradation, while previously there was around a 50% decrease between these two time points.

Similar to the previous experiment, P(DL)LA showed a different behavior in the first and second run of experiments. Despite having the same initial M_w in both experiments, in the second one polymer samples showed a faster degradation rate. Remarkably, sterile control samples degrade significantly faster than samples incubated in contaminated medium which might indicate that degradation could be diminished by the presence of bacteria. The change from transparent to opaque in early stages of incubation in these samples indicates higher water absorption. The rubbery appearance may imply that T_g of these samples was diminished below the temperature of incubation, changing from vitreous to amorphous state. In order to corroborate this hypothesis, T_g was measured by DSC for P(DL)LA samples incubated in sterile PBS and PBS contaminated with *Pseudomona* sp. (Annex B:). However, no changes were observed likely due to short incubation period. The effect of bacteria in this phenomenon is currently unknown, however it is possible that bacterial adhesion physically hinder water permeation.

3.2.3 Bacterial proliferation

Bacterial presence was observed for *P. aeruginosa* and environmental *Pseudomona* sp. during incubation period in all the polymers in study. But, remarkably *S. aureus* strain was not viable in the culture media containing only PBS and polymer. After only one day of incubation no viable colony of this specie grew in plate count agar for any of the polymers in study. The premature decrease in *S. aureus* concentration showed that this bacteria is more susceptible to hostile environments than the other species, which may be related to its gram positive nature in contrast with the gram negative nature of *Pseudomona* spp.

After two weeks of incubation, the initial concentration of 10^5 CFU/mL of *P. aeruginosa* increased over 10^6 CFU/mL. After that, the concentration of bacteria ranged between 10^6

and 10^7 CFU/mL (Figure 3-15). It was not recognizable a clear difference in the growth rate of bacteria between polymers. Also it was not observed any decrease in the concentration through time.

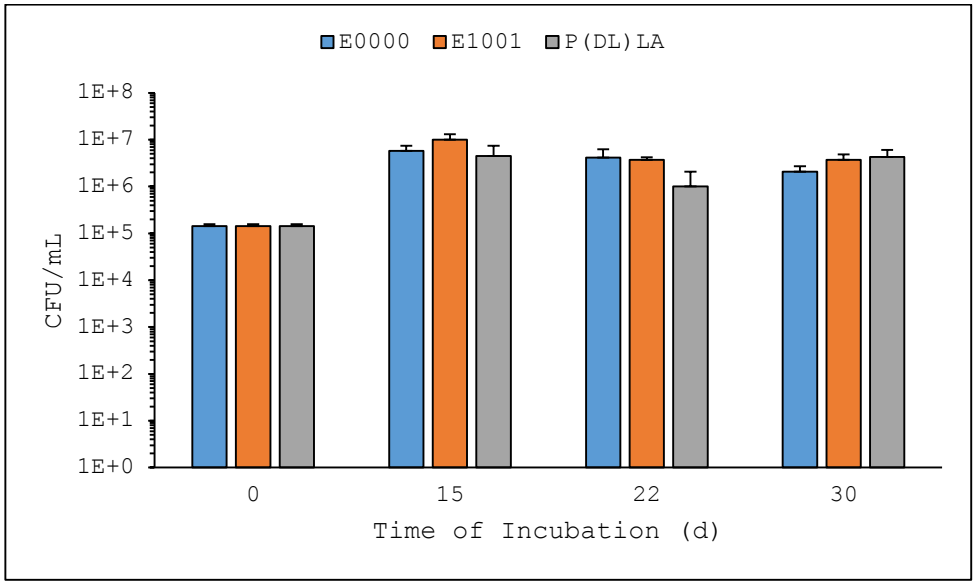

Figure 3-15: Concentration of *P. aeruginosa* increased from 10^5 to 10^6 CFU/mL during the first 2 weeks of incubation. Columns are presented as mean ± SD of three samples.

Growth of environmental *Pseudomona* sp. exhibited a similar behavior than the growth of *P. aeruginosa*. The initial concentration of 10^5 increased to 10^6 during the first 2 weeks, and then ranged between 10^5 and 10^6 CFU/mL (Figure 3-16).

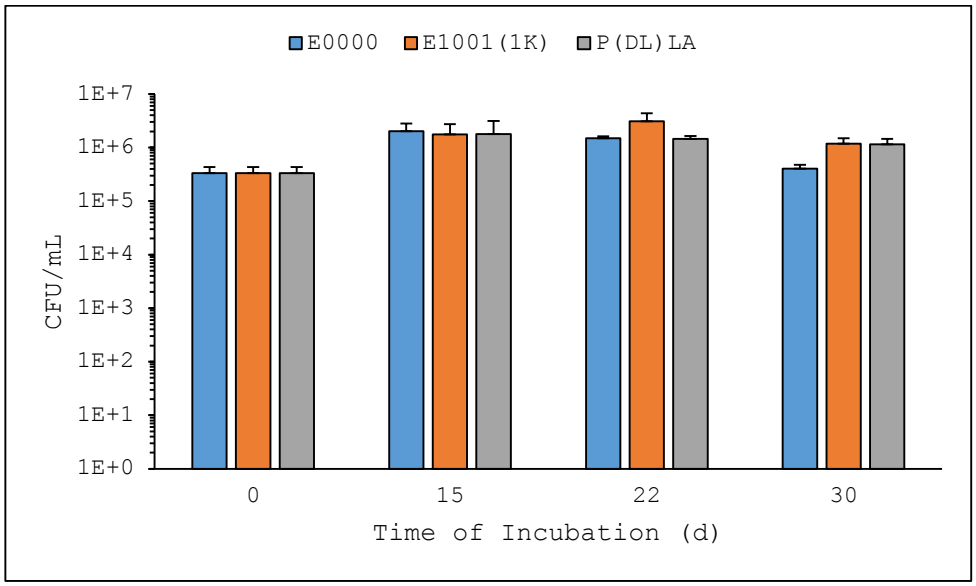

Figure 3-16: Concentration of environmental *Pseudomona* sp. increased from 10^5 to 10^6 CFU/mL during the first 2 weeks of incubation. Columns are presented as mean ± SD of three samples.

There was not observed a decrease in *P. aeruginosa* or environmental *Pseudomona* sp. concentration through time. After the initial increase in bacterial concentration, bacteria amount remained approximately constant. Nevertheless, it is expected that if the experiment continue, the concentration would decrease because of media nutrient depletion.

Additionally, it was not recognizable a clear difference in the growth rate of both bacteria in media containing different kinds of polymers. As polylactides have acidic degradation byproducts, it could be expected that their degradation process affect bacterial concentration. However, the results obtained in this study agree with Hofmann et al. (Hofmann et al., 1990), who reported no difference in the proliferation of *S. aureus*, *S. epidermidis*, *P. aeuginosa* and *E. coli* caused by the presence of PLA and its degradation byproducts *in vitro*, and Mainil-Varlet et al. (Mainil-Varlet et al., 2001) who showed that two polylactides with different degradation kinetics, P(L/DL)LA and P(L)LA, had the same proliferation of *S. aureus in vivo*.

3.3 Bacterial adhesion

Possible biofilms formation over polymer surface was observed by scanning electron microscope. E0000 films were incubated for 10 days with initial concentration of 10^5 CFU/mL and 10^8 CFU/mL of both *P. aeruginosa* and environmental *Pseudomona* sp. and the bacteria on the surface were observed under microscope at x2000 and x10000

P. aeruginosa subsisted for all the time of incubation, being clearly recognized under microscope (Figure 3-17). As expected, in the case of polymers incubated with an initial bacterial concentration of 10^5 CFU/mL it was observed a less amount of bacteria adhered to the polymer surface. However, it was possible to identify at higher magnification the secretion of substances that may indicate the formation of biofilm. In the case of *P. aeruginosa* with initial concentration of 10^8 CFU/mL the amount of bacteria observed was remarkably large. A closer look into the polymer surface in this condition allows observing more clearly that cells secreted a substance to interact with the polymer surface, indicating biofilm formation.

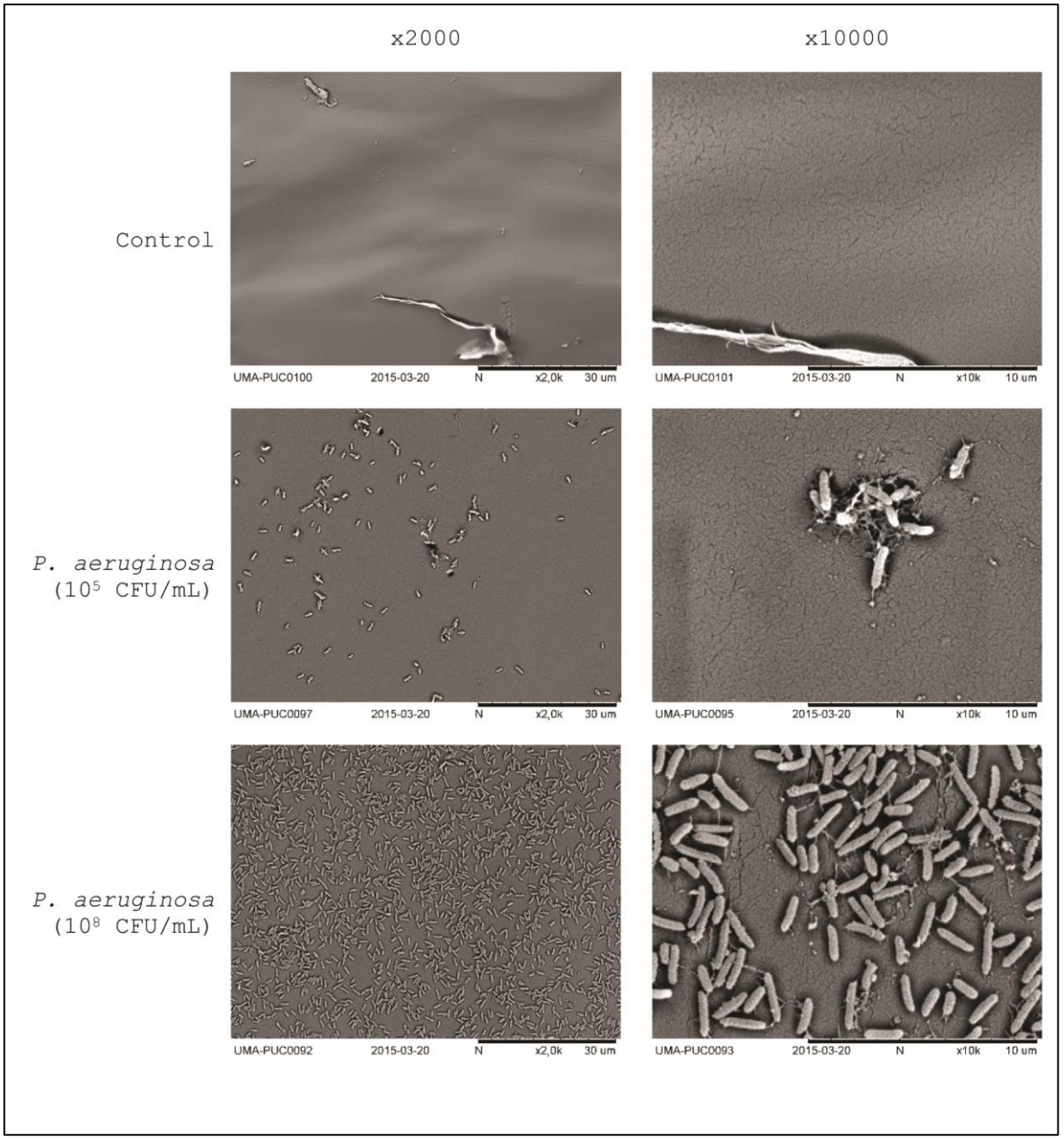

Figure 3-17: SEM photographs of E0000 surface incubated with *P. aeruginosa* at initial concentration of 10^5 and 10^8 CFU/mL at x2000 and x10000.

For films incubated in environmental *Pseudomona* sp., a less amount of bacteria adhered than *P. aeruginosa* was observed (Figure 3-18). At initial concentration of 10^5 CFU/mL it was only possible to observe isolated bacteria and little clusters. However, at initial concentration of 10^8 CFU/mL the interaction between bacterial cells was more evident, with a high secretion of substances probably associated with biofilm formation.

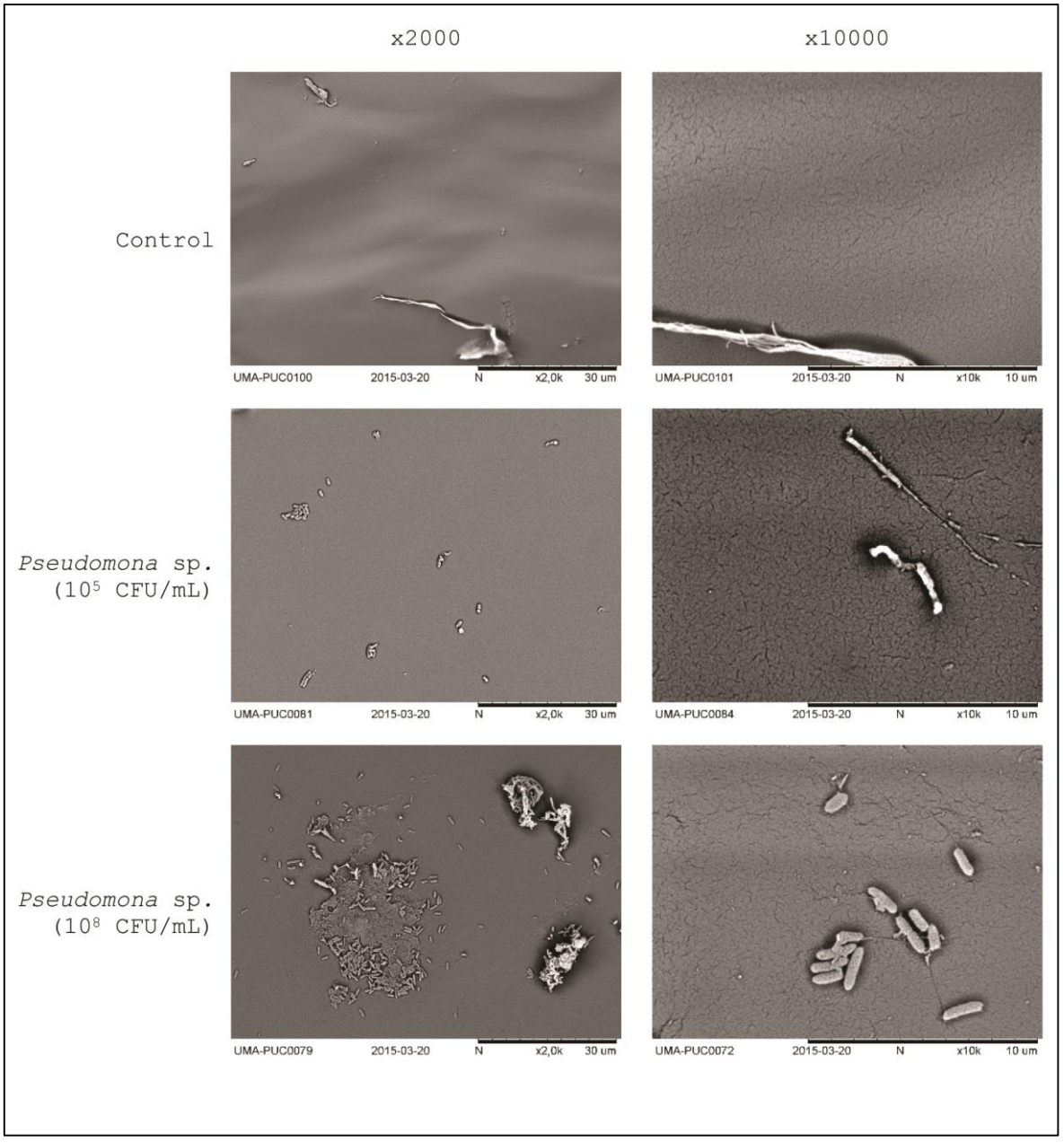

Figure 3-18: SEM photographs of E0000 surface incubated with environmental *Pseudomona* sp. at initial concentration of 10^5 and 10^8 CFU/mL at x2000 and x10000.

4. CONCLUSIONS

First, it was observed that the presence of environmental *Pseudomona* sp. in the culture media affects tyrosine-derived terpolymers degradation rate. Samples incubated in fibrinogen, which increases bacterial concentration, degrade significantly faster than samples incubated in PBS, in which there was less amount of bacteria. This may indicate that terpolymers undergoes enzymatic degradation by environmental *Pseudomona* sp. In the other hand, environmental *Pseudomona* sp. in culture media did not accelerate the degradation rate of poly(hydroxy acids), PLGA and P(DL)LA, indicating that these polymers did not suffer enzymatic bacterial degradation. Nevertheless, in the case of P(DL)LA it was observed a slight tendency to degrade slower in presence of bacterial contamination, related to water uptake differences, without current explanation.

Bacteria inoculated in sterile medium did not seem to affect terpolymers or poly(hydroxy acids) degradation. The possible cause of this phenomenon is that bacterial concentration under these circumstances was fewer than the bacterial concentration of spontaneous contamination. Future research could assess the minimum concentration of bacteria that affects terpolymer degradation and model the relationship between bacterial amount and M_w decrease.

Bacterial concentration remained approximately constant during all the incubation time for *P. aeruginosa* and environmental *Pseudomona* sp. but *S. aureus* did not survive beyond one day of incubation in this hostile environment, containing only sterile PBS and terpolymer sample, likely due to its gram positive nature.

The degradation process of the polymers in study has no effect in microbial growth rate. For terpolymers, which have biocompatible degradation products, it is not expected bacterial growth inhibition due to degradation. However, poly(hydroxyl acids) have acidic degradation byproducts which may alter bacterial proliferation. However, there were observed neither important changes in pH nor a diminished bacterial concentration.

Microscopic observation showed that *P. aeruginosa* and environmental *Pseudomona* sp. tend to adhere to polymer surface and secrete substances forming biofilms. For both species, the amount of adhered bacteria was increased when bacterial concentration was higher.

This work shows that bacteria affect tyrosine-derived terpolymers, accelerating their degradation rate, being capable to form biofilms on polymer surface. Therefore, it must be considered that bacterial contamination, before or after implantation, may promotes a premature collapse of terpolymer implants. Future research should be focus more deeply in terpolymer capacity to sustain bacterial adhesion, colonization and biofilm formation, key factors that trigger infections inside the patient's body, and also in their possible capacity to act as a carbon source promoting the growth of pathogenic bacteria.

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ANNEXES

ANNEX A: BIOINFORMATIC ANALYSIS FOR IDENTIFICATION OF BACTERIA RESPONSIBLE FOR SPONTANEOUS CONTAMINATION

Sequence analysis was performed using NCBI BLAST (http://blast.ncbi.nlm.nih.gov), specifically Standard Nucleotide BLAST (BLASTN), contrasting the query sequence with the nucleotide collection (nr/nt).

The sequence obtained with primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') had 1231 bp (Table A-1). When it was contrasted with BLAST databases, sequences with a coverage and identity of 99% were observed (Figure A-1). The best score was obtained by *Pseudomona indoloxydans* IPL-1, following by *Pseudomona pseudoalcaligenes* C70c.

From above, it was clearly observed that analyzed bacteria corresponds to *Pseudomona* genus. The particular species is not completely clear, since several hits were found. However, it is probable that the bacterium corresponds to either *Pseudomona indoloxydans* or *Pseudomona pseudoalcaligenes* species. Nevertheless, to obtain with precision species and strain of the bacterium, further analysis using specific *Pseudomona* primers should be performed.

Table A-1: FASTA format of sequence obtained with primer 1492R.

Sequence
>150411-10_M24_C9_1492R.ab1 1231
CCCGACTGAATCCTCCCGTGGGTACCGTCCCCCCGAAGGTTAGACTAGCTACTTCTGGAGCAACC
CACTCCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTGACATTCTGAT
TCACGATTACTAGCGATTCCGACTTCACGCAGTCGAGTTGCAGACTGCGATCCGGACTACGATCG
GTTTTATGGGATTAGCTCCACCTCGCGGCTTGGCAACCCTTTGTACCGACCATTGTAGCACGTGT
GTAGCCCTGGCCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCGGTTTGTCACCG
GCAGTCTCCTTAGAGTGCCCACCATTACGTGCTGGTAACTAAGGACAAGGGTTGCGCTCGTTACG
GGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCAGCACCTGTGTCTGAGTTCC
CGAAGGCACCAATCCATCTCTGGAAAGTTCTCAGCATGTCAAGGCCAGGTAAGGTTCTTCGCGTT
GCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAAC
CTTGCGGCCGTACTCCCCAGGCGGTCAACTTAATGCGTTAGCTGCGCCACTAAGTTCTCAAGGAA
CCCAACGGCTAGTTGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCC
ACGCTTTCGCACCTCAGTGTCAGTATCAGTCCAGGTGGTCGCCTTCGCCACTGGTGTTCCTTCCT
ATATCTACGCATTTCACCGCTACACAGGAAATTCCACCACCCTCTACCGTACTCTAGCTCGCCAG
TTTTGGATGCAGTTCCCAGGTTGAGCCCGGGGCTTTCACATCCAACTTAACGAACCACCTACGCG
CGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGCACGA
AGTTAGCCGGTGCTTATTCTGTCGGTAACGTCAAAACAGCAAGGTATTAACTTACTGCCCTTCCT
CCCAACTTAAAGTGCTTTACAATCCGAAGACCTTCTTCACACACGCGGCATGGCTGGATCAGGCT
TTCGCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAATCTGGACCGTGTCTCAGTTCC
AGTGTGACTGATCATCCTCTCAAACCAGTTACGGATCCTTCCCCTTGGTGAGCCATTCCTT

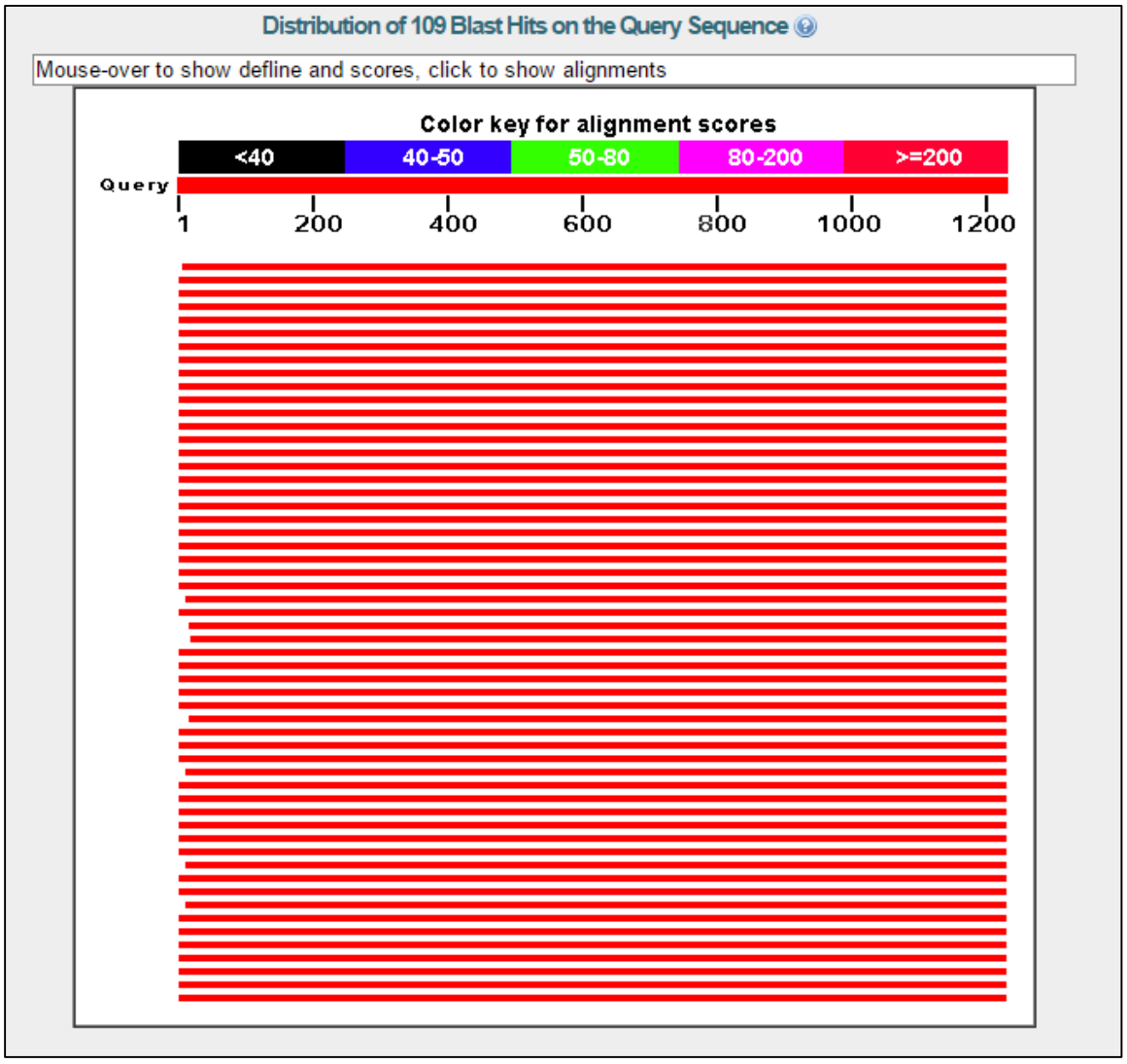

Figure A-1: BLAST results obtained for the analyzed sequence. High coverage and identity (99%) was obtained for several species.

ANNEX B: GLASS TRANSITION TEMPERATURE OF P(DL)LA SAMPLES STUDIED BY DIFFERENTIAL SCANNING CALORIMETRY

P(DL)LA samples incubated in sterile PBS degraded faster than samples incubated in a contaminated medium. Additionally, it was observed that polymer samples in sterile medium acquired an opaque and rubbery appearance before than non-sterile ones. The loss of transparency indicates a higher amount of water uptake. Additionally, the rubbery appearance could be related to a reduction of their T_g under 37°C, which implies that these polymers were in rubbery state under experimental conditions which triggers a faster degradation. In order to corroborate this phenomenon, P(DL)LA samples were incubated for 6 hours in two conditions: (i) sterile PBS or (ii) PBS inoculated with 10^8 CFU/mL approx. of environmental *Pseudomona* sp. From DSC analysis of the first run it was observed that wet T_g was above 37°C, being approximately 42°C for both polymers.

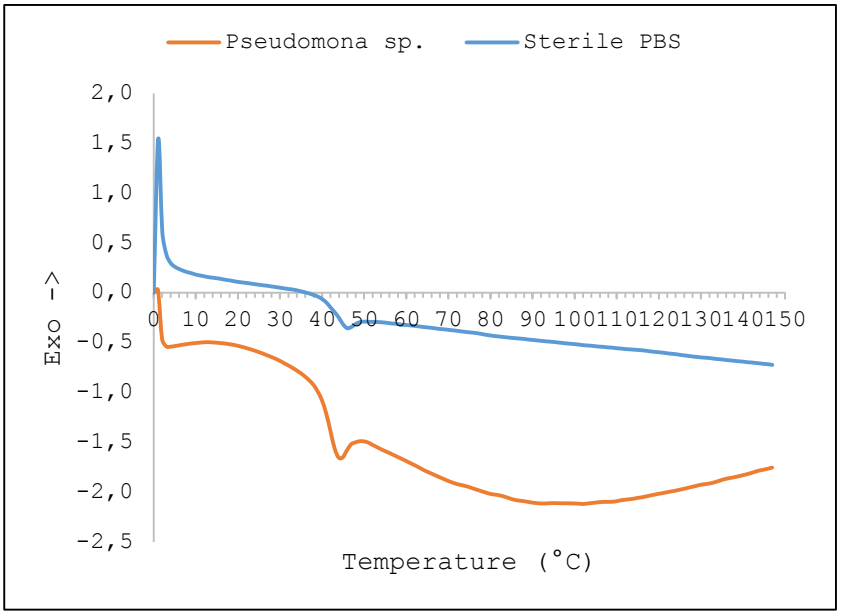

Figure B-1: First run DSC of P(DL)LA samples incubated in sterile PBS (blue line) or PBS with *Pseudomona* sp. (orange line).

From DSC analysis of the second run, it was observed that dry T_g of samples incubated in contaminated medium was higher (48.29°C) than samples incubated in sterile PBS (42.11°C).

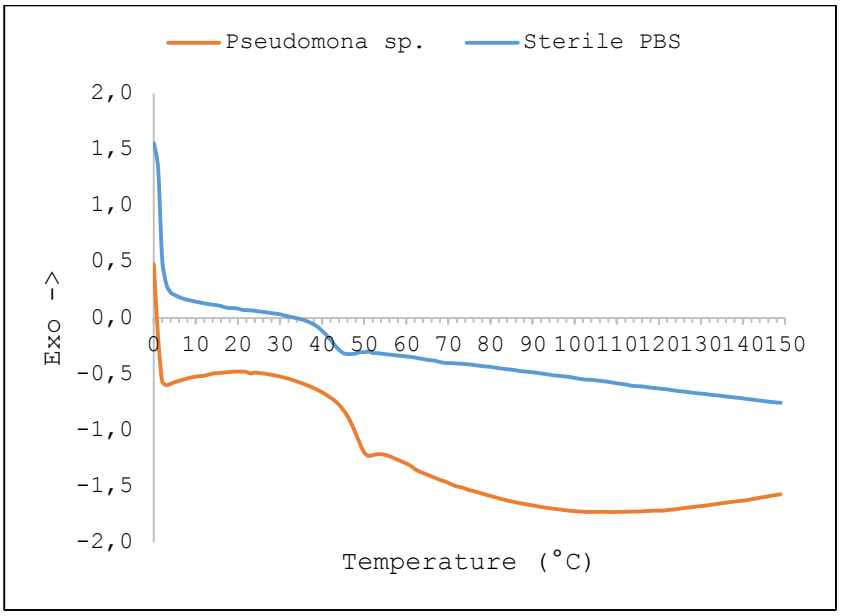

Figure B-2: Second run DSC of P(DL)LA samples incubated in sterile PBS (blue line) or PBS with *Pseudomona* sp. (orange line).

After 6 hours of incubation, water absorption was very low. It is expected that samples incubated for more time absorb greater amounts of water, and therefore its T_g will decrease over time. Further analysis with samples incubated for longer time span must be done in order to corroborate this presumption.

ANNEX C: RELATIONSHIP BETWEEN PHYSICAL PROPERTIES AND BIOLOGICAL RESPONSE IN TYROSINE-DERIVED POLYARYLATES EXPLORED BY ASSOCIATION RULES

This work was presented in:

Daniela C. Soto, Loreto M. Valenzuela. Relationship between physical properties and biological response in tyrosine-derived polyarylates. XXIII International Material Research Congress, Cancún, México. Agosto, 2014. *(Oral presentation)*

Daniela C. Soto, Loreto M. Valenzuela. Relationship between physical properties and biological response in tyrosine-derived polyarylates explored by association rules. XXVI Annual Conference of the European European Society for Biomaterials. Liverpool, Reino Unido. Agosto, 2014. *(Poster presentation)*

Introduction

Polymers are widely used in several medical applications, from degradable scaffolds to drug delivery systems. Nowadays the focus is to design polymers with specific properties for each application (Kohn, 2004). To achieve this goal, it is necessary to understand relationships between polymer chemistry, physical properties (glass transition temperature [T_g] and air-water contact angle), behavior in physiological environment (degradation, erosion and water uptake) and biological response (protein adsorption and cell adhesion-proliferation).

Combinatorial chemistry has enabled the generation of polymer libraries, which are large collections of polymer structures created using molecular modeling tools (Kohn, 2004). One interesting example is the library of L-tyrosine derived polyarylates (Figure C-1) synthesized by Kohn and coworkers (Fiordeliso, Bron, & Kohn, 1994). Several techniques have been used to build discrete (Abramson, Alexe, Hammer, & Kohn, 2005) and numerical models (Gubskaya, Kholodovych, Knight, Kohn, & Welsh, 2007; Smith, Kholodovych, Knight, Welsh, & Kohn, 2005) for this polymer library. These studies only correlate the physical

properties and chemical structure of polymers with protein adsorption or cell response separately.

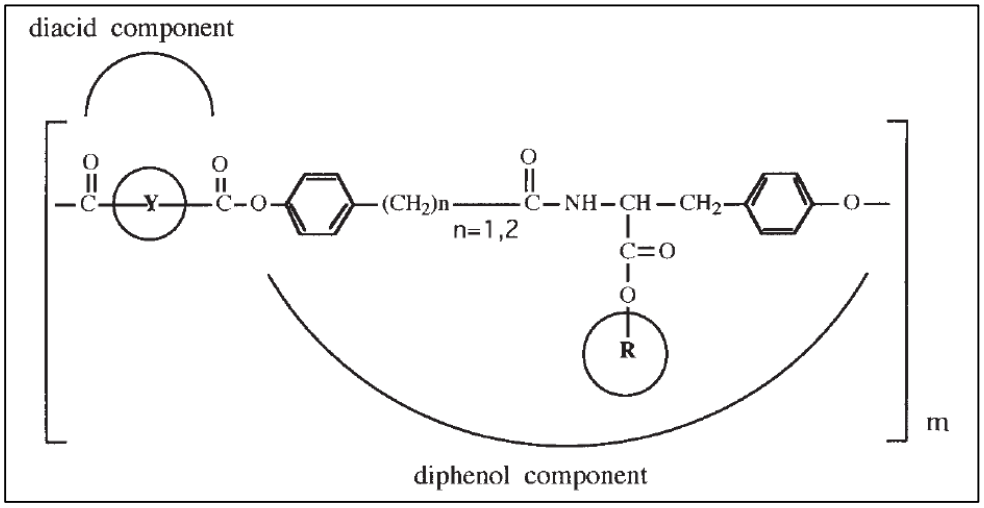

Figure C-1: Combinatorial library of L-tyrosine derived polyarylates is composed of 112 polymers synthetized using 8 diacid units and 14 diphenols units (Abramson, Alexe, Hammer, & Kohn, 2005)

In this study we used association rules to find relevant patterns between all of these properties jointly on the L-tyrosine derived polyarylates library. Knowing these relationships would guide future experimental research, making easier to find polymers with desired properties.

Methods

The analysis of the polymer database included several steps (Figure C-2). First, the dataset of polymer properties was organized. We used a polymer library of 112 polyarylates created by Kohn and coworkers (Fiordeliso, Bron, & Kohn, 1994). The information available include physical properties, such as T_g, contact angle, water uptake and degradation half time (Table C-1), and biological properties such as protein adsorption and normalized metabolic activity (NMA) of cells seeded in these polymers (Table C-2). The information of T_g and water-air contact angle were available for all polymers in the library, but the other properties were only described for a fraction of the database, being only 5 polymers fully

characterized. For water uptake and degradation, predictions obtained by Valenzuela et al. were included (Valenzuela, Knight, & Kohn, 2012).

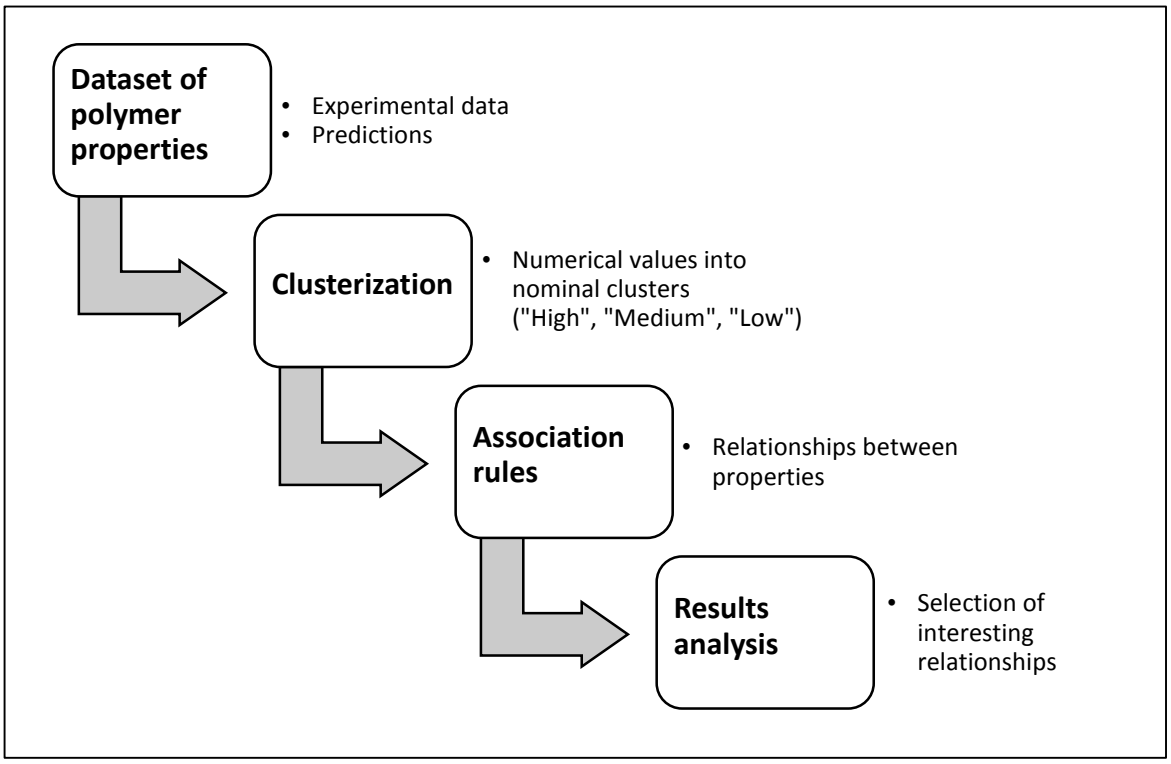

Figure C-2: Scheme of the steps in the data analysis performed. First, the dataset was manually organized including experimental data and predictions. Second, numeric data were assigned to clusters. Then, association rules were found and analyzed.

Table C-1: Physical properties of L-tyrosine derived polyarylates obtained from literature (*exp*: experimental data, *pred*: predicted data).

Physical Properties	Quantity	Reference
T_g	112 exp	(Smith, et al., 2004)
Contact angle	112 exp	(Smith, et al., 2004)
Water uptake % (28 d)	23 exp 23 pred	(Valenzuela, Knight, & Kohn, 2012)
Degradation half time	23 exp 22 pred	(Valenzuela, Knight, & Kohn, 2012)

Table C-2: Biological properties of L-tyrosine derived polyarylates obtained from literature.

Biological properties	Quantity	Reference
Fibrinogen adsorption	45 exp	(Weber, Bolikal, Bourke, & Kohn, 2004)
Albumin adsorption	41 exp	(Abramson, Alexe, Hammer, & Kohn, 2005)
Rat lung fibroblast NMA	69 exp	(Abramson, Alexe, Hammer, & Kohn, 2005)
Foreskin fibroblast NMA	94 exp	(Abramson, Alexe, Hammer, & Kohn, 2005)

Second, the values of properties were clustered into "high", "medium" or "low" class. The procedure was done in data-mining software WEKA (Waikato Environment for Knowledge Analysis) (Hall, et al., 2009) using Expectation-Maximization algorithm (Dempster, Laird, & Rubin, 1977).

Finally, association rules were obtained in order to find patterns in the dataset. This methodology recognizes when certain conditions occurred simultaneously. We set the minimum support as 9% (i.e., a rule must contain a pattern present at least in 90% of the polymers), and the confidence as 90% (i.e., the presence of certain condition(s) imply the presence of other condition(s) in at least 90% of the cases). For example, for a rule A + B → C, the attributes A, B and C must appear together at least 10 times and in 90% of the cases in which A and B were together, also C must be present. This procedure was also performed in WEKA.

Results and discussion

First, it was observed that T_g increase is associated with a decrease in contact angle (R^2=0.61) (Figure C-3). This tendency was reported previously by Smith et al. (Smith, et al., 2004), which corroborates that the present approach was capable to capture relationships previously observed.

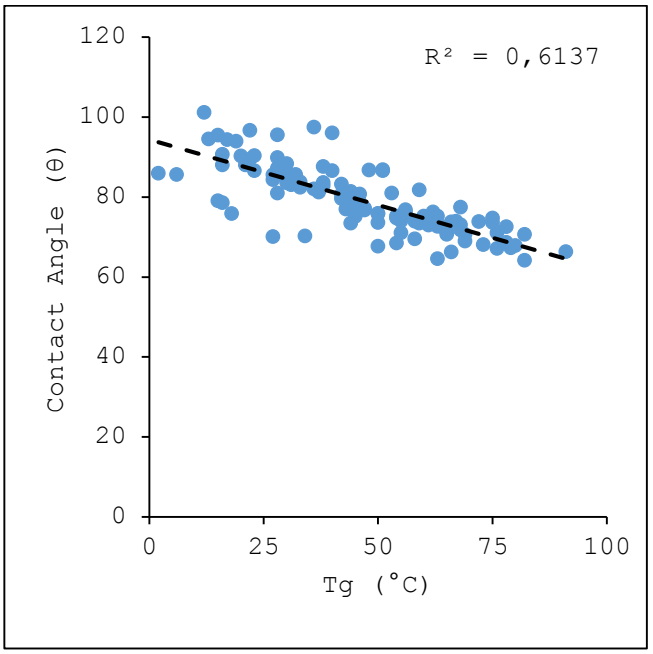

Figure C-3: Contact angle and T_g of L-tyrosine derived polyarylates relationship, i.e., while T_g grows, contact angle diminishes. (R^2=0.61)

In general, it is expected that water uptake and degradation rate are related in polymers that undergo hydrolytic degradation. However, it was not found a clear correlation between these two variables in the whole polyarylates database. But when only high contact angle polymers where considered, it was observed that fast degradation (i.e., low degradation half time) was related with low water uptake (Figure C-4). On the other side, for polymer with low contact angle it was observed that a low degradation, i.e., high degradation half time, is related with low water uptake.

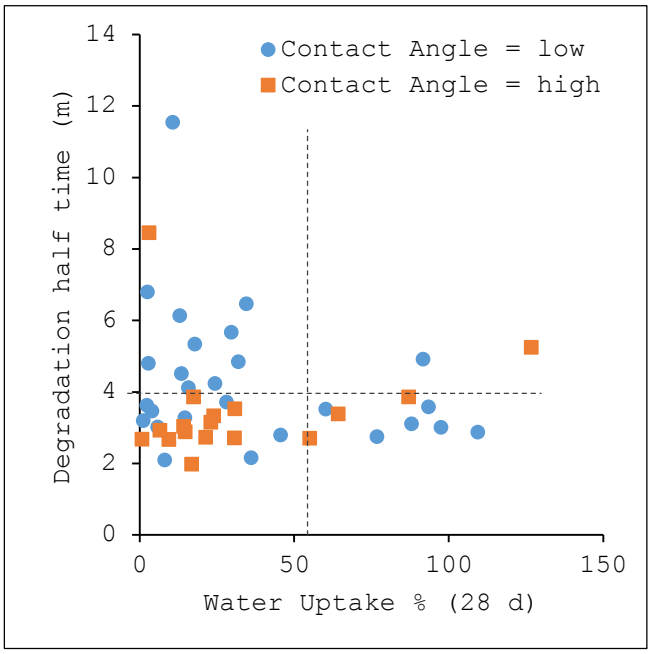

Figure C-4: Polyarylates with a high contact angle and low water uptake have a fast degradation, but polyarylates with a low contact angles and low water uptake showed a low degradation.

In terms of biological properties, two relevant relationships were found. First, it was observed a slight tendency to have a high normal foreskin fibroblast NMA when adsorbed fibrinogen was high. Nevertheless, this tendency was more notorious when only polymers with a high T_g were considered (Figure C-5). Second, it was observed that polymers with a high fibrinogen adsorption and a high rat lung fibroblast NMA are usually associated with a fast degradation (Figure C-6).

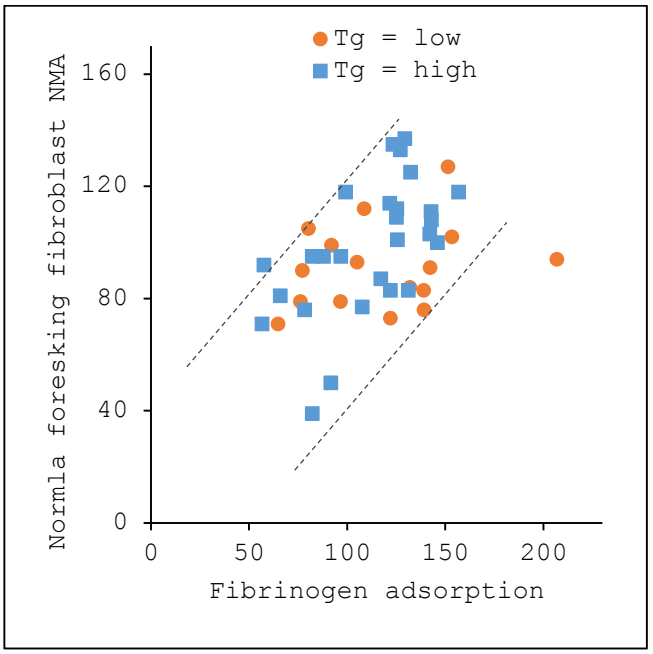

Figure C-5: Fibrinogen adsorption and normal foreskin fibroblast metabolic activity are slightly related. However, when only polymers with a high T_g are considered the correlation is more notorious.

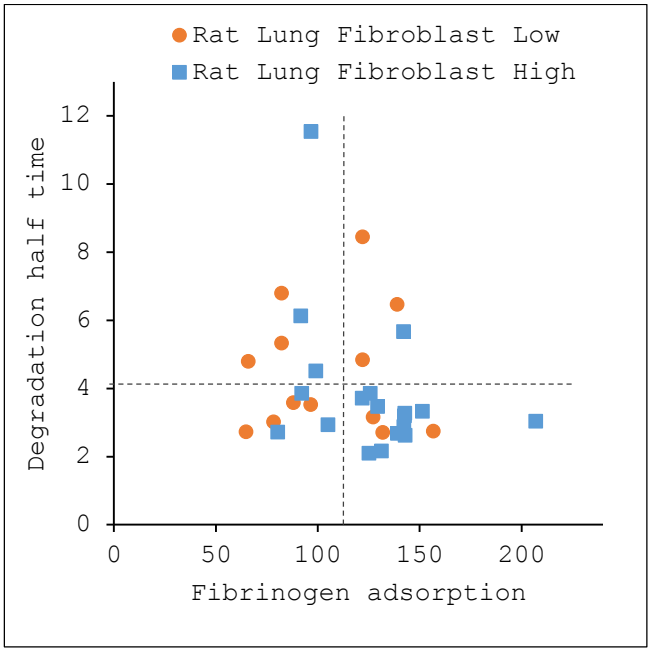

Figure C-6: Polymers with a high fibrinogen adsorption and a high at lung fibroblast NMA are usually associated with a fast degradation (low degradation half time).

Conclusions

In the present work, relationships between different properties of a set of polymers were found through association rules analysis. Filtering relevant rules is the most challenging task in this methodology. Having the complete set of experimental values for all polymers, or a prediction of them, will allow more and better association rules that will in turn allow a better selection of polymers for different applications.

Besides having an incomplete dataset of properties, it was possible to find some relevant association rules. It was observed that contact angle and T_g are inversely related, which was already reported in literature. Additionally it was found that relationships between water uptake and degradation rate are more notorious if the dataset is divided in terms of physical properties such as contact angle. Similarly, relationships between fibrinogen adsorption and metabolic activity were more notorious when only high T_g polymers were considered.

These results showed that hidden relationships between properties emerge when three variables are analyzed jointly. Analyzing these relationships may help to guide further research and to develop integrated models that included physical and biological properties.

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