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BIOMATERIALS

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Chapter 1

Synthesis and Characterization of Functional Nano/Micro-Particles for Antibacterial & Antibiofouling Applications

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Abstract

The present study describes the engineering of functional antibacterial nano/micro-particles and their utilization for antibiofouling applications. For this purpose, two new vinylic monomers were synthesized, isothiouronium methyl styrene (ITMS) and methyl styrene farmin (MSF). Isothiouronium salts are well known for their antibacterials activities. The MSF monomer contains quaternary ammonium and long alkyl chain groups, and therefore is expected to possess efficient antibacterial and antibiofilm properties. The use of polymers is expected to enhance the efficacy of some existing antibacterial agents. It is well known that the incorporation of the monomers into polymeric particles significantly reduces their toxicity. The monomers were then polymerized by dispersion co-polymerization mechanism to form crosslinked particles. Here, we describe the synthesis and properties of three kinds of cross linked particles; polyisothiouronium methyl styrene (PITMS) microparticles, polyisothiouronium methyl styrene nanoparticles (PITMS NPs) and poly(methyl styrene farmin) nanoparticles (PMSF NPs). The effect of various polymerization parameters on the diameter and size distribution of the formed nano/microparticles has been elucidated. The antibacterial activity of the optimal nano/micro-particles was illustrated for two types of Gram-negative and two types of Gram-positive bacteria pathogens; *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Listeria innocua*, respec-

tively. Polyethylene terephthalate films were coated with a thin layer of the PITMS and PMSF NPs and their antibiofouling properties was demonstrated. The formed films reduced the viability of *Listeria* biofilm, therefore making them an excellent candidates for non-fouling surfaces. These new NPs could be utilized in a variety of industrial applications, as a new type of antibacterial agent and antibiofilm additive, due to their promising antibacterial and antibiofilm properties.

Introduction

Bacterial infections are one of the major global concerns in the past decades and are the main cause of infectious diseases, due to the growing resistance of bacteria to common antibiotics, both in Gram-negative and Gram-positive bacteria. This has led to much attention, both in academic and industry research, for the development of new antibacterial agents to be used in different fields such as treatment of water, sanitation, food, medicine, etc [1–4].

Antibacterial activity is related to compounds that locally kill microorganisms or inhibit their growth, without being toxic to the surrounding tissue. Common antibacterial agents include silver NPs [5,6], antibacterial peptides [3,7], phosphonium salts [7,8], isothiouronium compounds [9–12] and quaternary ammonium compounds [13–15]. The isothiouronium and quaternary ammonium compounds mechanism is different from the mechanism of the classical antibiotics, and the bacteria exhibits a prolonged sensitivity against them [1,14].

Thiourea, isothiouronium compounds and their derivatives, constitute an important class of compounds, exhibit a wide range of antibacterial activities and play an important role in many chemical and biological processes [9–12]. The isothiouronium terminal amino functional group favors binding to peptide terminating with acyl-D-alanyl-D-alanine (Ac-D-Ala-D-Ala). Antibacterial activity of isothiouronium compared to thiourea compounds (which lack positively charged N-terminus group) have 10-fold grater binding constant to Ac-D-Ala-D-Ala, in a bacterial cell-wall model, due to the enhanced acidity of the NH moieties [16].

Another important class of antibacterial agents are the positively charged quaternary ammonium compounds [13–15]. The bacterial cellular membrane is mainly composed of negatively charged phospholipids. The positively charged quaternary ammonium compounds strongly interact with the phospholipids and cause damage to the bacterial cell membrane [3,4,13,17,18]. Furthermore, quaternary ammonium compounds containing an alkyl chain group cause damage to the cell membrane through hydrophobic and electrostatic interactions with the hydrophobic lipid membrane. The antibacterial activity is improved as the length of the hydrophobic chain increases [19,20].

Low molecular weight antibacterial agents are toxic to both the environment and humans, due to the high dosage required for treatment. This has prompted the development of alternative antibacterial agents that overcome the intolerable toxicity. Polymer molecules, bearing antibacterial functional groups, are commonly used to over-

come problems associated with the low molecular weight antibacterial agents. Polymeric antibacterial agents reduce the residual toxicity, increase their efficiency and selectivity, and prolong the lifetime of the antibacterial agent [21]. They are also non-volatile, not harmful to the environment, chemically stable and have low permeability through the skin [8,13,14,18,21–23]. Furthermore, polymers in the form of nano/micro-particles have advantages as antibacterial agents due to their high surface-to-volume ratio, resulting in enhanced antibacterial activity [17,24].

Polymeric particles may be synthesized in a single step by dispersion radical polymerization mechanism. Prior to the polymerization, the monomer, initiator and stabilizer are dissolved in the homogeneous polymerization medium [25–27]. With the initiation of the polymerization, the initiator radicals react with the monomer molecules to form oligomeric radicals, which at a critical chain length precipitate from the solution as small nuclei. This step is followed either by agglomeration of small nuclei, polymerization of monomer in the swollen nuclei, or by seeded polymerization on the nuclei surfaces, leading to the growth of the particles to their final size. During the growth process, the stabilizer is adsorbed on the surface of the particles and prevents agglomeration. Termination occurs either when all of the monomer molecules have polymerized or when the stabilizer is adsorbed on the particles' surface, and forms a relatively packed coating, which does not allow further swelling of the formed particles [25–27]. The monomer concentration, weight

ratio of the monomers, initiator concentration, stabilizer type and concentration, as well as the solvent type, have an important role in determining the size of the formed particles [25].

Adhesion and subsequent growth of bacteria on surfaces cause the formation of biofilm. There is a growing demand for reliable antibacterial surfaces that can effectively minimize bacterial colonization [28]. Quaternary ammonium salts are widely used as 'cationic disinfectants' and antibacterial coating to minimize the problems of biofouling [29,30].

Listeria monocytogenes, is a gram-positive bacterium, that have the ability to survive in the form of biofilm and therefore is highly resistant in extreme conditions. The organism is recognized as a food-borne pathogen and causes listeriosis in humans. In order to reduce the outbreaks of listeriosis and decrease economic losses to the food industry, an effective method of preserving foods from the effect of bacterial growth is by adding antibacterial agents [31,32].

In this study we present the synthesis of nano/micro-particles based on isothiouronium compounds and quaternary ammonium salts for antibacterial and antibiofouling applications. Two antibacterial vinylic monomers were synthesized: 1. isothiouronium methyl styrene (ITMS), containing isothiouronium group, 2. methyl styrene farmin (MSF), containing a quaternary ammonium groups with a long alkyl chain. In addition, the synthesis

and characterization of three types of antibacterial particles is described; poly (isothiouronium methyl styrene) (PITMS) microparticles, polyisothiouronium methylstyrene nanoparticles (PITMS NPs) and poly(methyl styrene farmin) nanoparticles (PMSF NPs). The effect of various polymerization parameters on the diameter and size distribution of the produced nano/micro-particles was studied. The antibacterial activity of the formed particles was evaluated using two types of Gram-negative and two types of Gram-positive bacteria; *Escherichia coli* (E. coli), *Pseudomonas aeruginosa* (P. aeruginosa), *Staphylococcus aureus* (S. aureus) and *Listeria innocua,* respectively. The potential use of the PITMS and PMSF NPs as an inhibitor of biofilm formation was demonstrated using Listeria as a model. These new nano/micro-particles have shown the ability to kill bacteria and prevent biofilm formation. Therefore, they are potential candidates to be used in a variety of industrial applications, as a new type of antibacterial agents and antibiofilm additives.

Synthesis of the ITMS Monomer

The monomer ITMS was synthesized according to the literature (Figure 1) [33]. Briefly, thiourea (0.21 mol) was dissolved in methanol (60 mL), followed by the addition of *p*-chloromethyl styrene (CMS, 0.2 mol) to the solution. The reaction mixture was stirred at room temperature for 24 h. Diethyl ether was then added to precipitate the desired ITMS monomer. The filtered product was purified by dissolving it in ethanol and re-precipitation with ether

(90%). The ITMS monomer was characterized by ^{1}H and ^{13}C NMR and mass spectra [34].

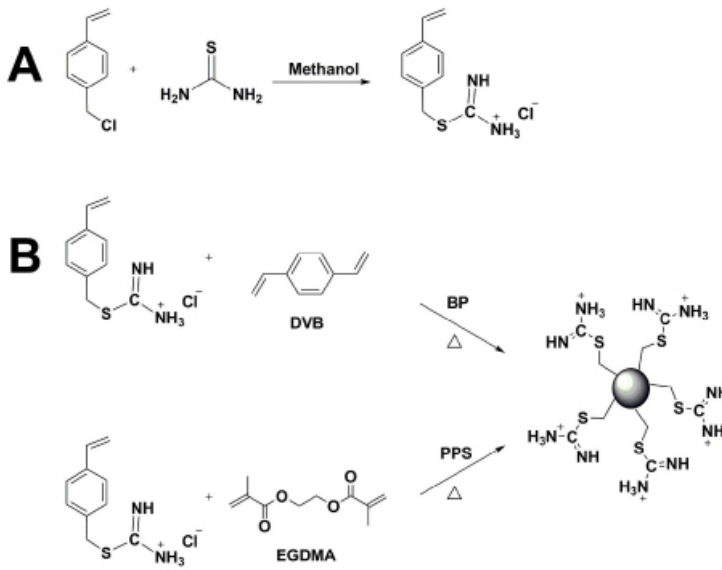

Figure 1: Synthetic scheme of the ITMS monomer (A) and PITMS nano/micro-particles (B).

Synthesis and Characterization of the PITMS Microparticles

Functional PITMS microparticles were prepared by dispersion co-polymerization of ITMS (450 mg) and divinylbenzene (DVB) (50mg), with benzoyl peroxide (BP) (25 mg) as the initiator and polyvinylpyrrolidone (PVP, Mw 360,000) (100mg) as the stabilizer in 2-methoxyetha-

nol (10 mL). The mixture was shaken at 73 °C for 22 h. The particles were washed for removal of excess reagents by intensive centrifugation cycles with ethanol and water. The effect of various polymerization parameters on the diameter and size distribution, and the polymerization yield of the PITMS microparticles were studied [34].

The yield of the crosslinked PITMS microparticles was calculated to be 85%. The dry diameter of 380 ± 40 nm is demonstrated by the SEM image (Figure 2A) and the hydrodynamic diameter of 440 ± 50 nm is evaluated by the dynamic light scattering histogram (Figure 2B). The hydrodynamic diameter is slightly larger than the dry diameter since it also takes into account Brownian motion, absorbed and surface-adsorbed solvent or water molecules [35].

Zeta potential (ζ–potential) measurements are commonly used to study the stability of particles dispersed in aqueous solution. PITMS microparticles have a positively-charged surface which could create repulsion between particles, thereby preventing aggregation. PITMS microparticles demonstrate a slight decrease in the ζ–potential of the particles when increasing the pH of the aqueous continuous phase from 2 to 10. A further increase in the pH from 10 to 12, leads to a sharp decrease in the ζ–potential of the microparticles. In an acidic environment, the particle surface possesses positively charged isothiouronium groups, and as the pH increases, the surface charge decreases. At the isoelectric point (around pH 11), the

particles are not stable due to aggregation. Increasing the pH of the continuous phase above 11.5 is likely to cause hydrolysis of the isothiouronium groups involving deprotonation of the thiol groups, as indicated by the literature [36,37]. (Figure 2C)

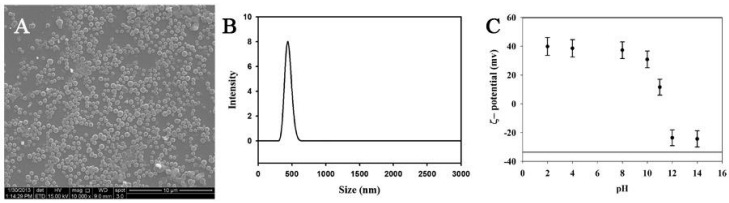

Figure 2: SEM image (A), hydrodynamic size histogram (B) and ζ-potential as a function of pH (C) of the PITMS microparticles.

Synthesis and Characterization of the PITMS NPs

Functional PITMS NPs were prepared by dispersion co-polymerization of ITMS (425 mg) and ethylene glycol dimetacrylate (EGDMA) (75 mg), with potassium persulfate (PPS) (25 mg) as the initiator and Tween 20 (100 mg) as the stabilizer in water (10 mL). The mixture was shaken at 73 °C for 15 h. The obtained NPs dispersed in water were then isolated from impurities by dialysis. The formed PITMS NPs were washed with water at 60 °C in order to remove excess reagents. The effect of various polymerization parameters on the diameter and size distribution, and the polymerization yield of the PITMS NPs were studied [38].

The yield of the crosslinked PITMS NPs was calculated to be 75%. The dry diameter of 19 ± 2 nm is demonstrated by the TEM image (Figure 3A) and the hydrodynamic diameter of 67 ± 8 nm is evaluated by the dynamic light scattering histogram (Figure 3B). The ζ–potential of the PITMS NPs exhibit a consistent sharp decrease in the ζ–potential as the pH increases from pH 4.0 to 10.5. At the isoelectric point (around pH 10.2), the particles are not stable, due to possible aggregation. Increasing the pH of the continuous phase above 11.5 probably causes hydrolysis of the isothiouronium groups into deprotonated thiol groups, as reported in the literature [39] (Figure 3C).

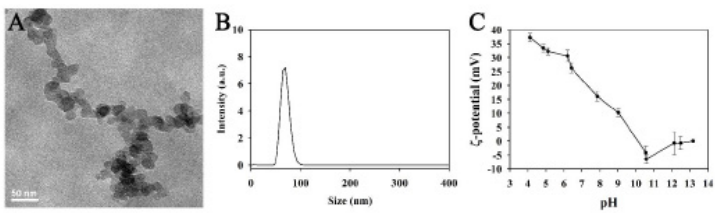

Figure 3: TEM image (A), hydrodynamic size histogram (B) and ζ-potential as a function of pH (C) of the PITMS NPs.

Synthesis of the MSF Monomer

The new MSF monomer was synthesized by dissolving sodium carbonate (0.33 mmol) in water (5.5 mL). Farmin (17 mmol) was then added under a nitrogen atmosphere and the mixture was heated to 68 °C for 1 h. Then, *p*-chloromethyl styrene (CMS) (20 mmol) was added and the mixture was stirred until a clear solution was obtained (Figure 4). Phase separation was performed by

dichloromethane and the desired MSF monomer was precipitated by evaporation of the solvent (92%). The MSF monomer was characterized by ^{1}H NMR, TOF MS+ and FTIR [40].

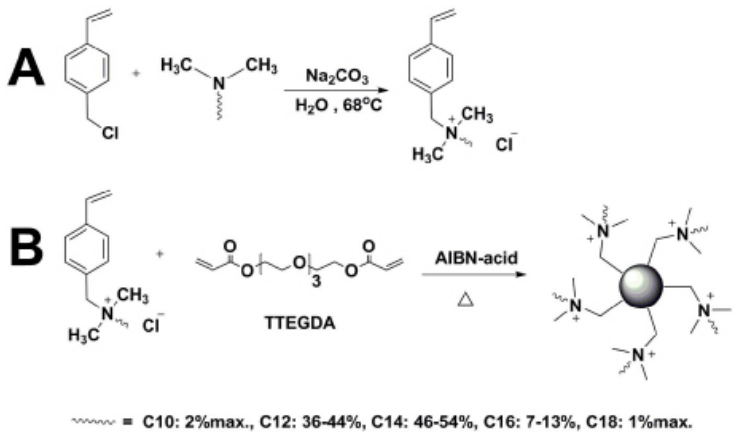

Figure 4: Synthetic scheme of the MSF monomer (A) and PMSF NPs (B).

Synthesis and Characterization of the PMSF NPs

PMSF NPs were prepared by dispersion co-polymerization of MSF (237.5 mg) and tetra (ethylene glycol) diacrylate (TTEGDA) (12.5 mg), with 4,4'- azobis (4-cyanovaleric acid) (AIBN-COOH) (12.5 mg) as the initiator and PVP (25 mg) as the stabilizer in water (5 mL). The mixture was purged with N_2 to exclude air and then shaken at 80 °C for 24 h. The NPs were purified from excess reagents by extensive dialysis cycles against distilled water. The ef-

fect of various polymerization parameters on the diameter and size distribution of the PMSF NPs was studied [40].

The yield of the crosslinked PMSF NPs was calculated to be 75%. The dry diameter of 40 ± 9 nm is demonstrated by the TEM image (Figure 5A) and the hydrodynamic diameter of 139 ± 17 nm is evaluated by the dynamic light scattering histogram (Figure 5B). The ζ-potential of the PMSF NPs is not affected when increasing the pH from 4.5 up to 7.7, indicating a very stable dispersion. Increasing the pH up to 12.1 leads to a sharp decrease in the ζ-potential, probably attributed to the increasing concentration of non-charged groups. At a pH higher than 12.1, as the ζ-potential approaches the isoelectric point, the NPs are totally agglomerated (Figure 5C).

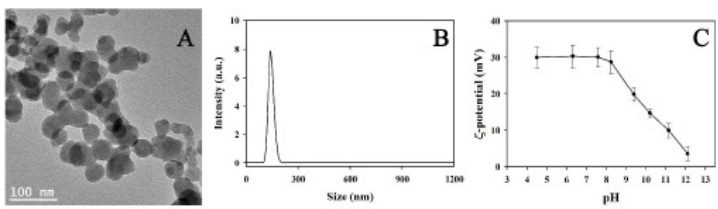

Figure 5: TEM image (A), hydrodynamic diameter histogram (B) and ζ-potential as a function of pH (C) of the PMSF NPs.

Antibacterial and Antibiofilm Assays

Bacterial Cultures and Growth Conditions

The Gram-negative bacteria *E. coli* ATCC 8739 and *P. aeruginosa* PAO1, and the Gram-positive bacteria *S. aureus* FRF1169 and Listeria ATCC 33090 were grown over-

night either in Luria Bertani (LB, Difco), Nutrient Broth (NB, Sigma) or Brain Heart (Difco) growth mediums at 37 °C under agitation (250 rpm). On the following day, the overnight cultures were each diluted into 2-fold concentrated medium to obtain a concentration of $2x10^5$ colony-forming units (CFU/mL).

Antibacterial Activity Assay of the Nano/Micro-Particles

Antibacterial Activity of the PITMS Nano/Micro-Particles

The antibacterial activity of the PITMS microparticles and PITMS NPs was evaluated by treatment overnight of the bacterial suspensions with various concentrations of PITMS microparticles (1, 0.5, and 0.25%), PITMS NPs (0.5, 0.25 and 0.125%) or sterilized water (control). The following day, ten-fold serial dilutions were carried out, and the bacterial cells were plated on LB agar plates, followed by their incubation at 37 °C for 20 h. Cell growth was monitored and determined by viable cell count and expressed as colony-forming units (CFU/ml).

The PITMS microparticles (1 and 0.5%) and PITMS NPs (0.5 and 0.25%) with each of the four bacterial strains, resulted in total killing of all the bacteria, compared to the negative control. However, only partial bactericidal activity was observed at concentrations of 0.25 and 0.125% of the PITMS microparticles and NPs, respectively. The minimum inhibitory concentration (MIC) for the PITMS

microparticles and NPs was found to be 0.5 and 0.25%, respectively (Table 1).

These results indicate that the PITMS nano/microparticles are active against both Gram-negative and Gram-positive bacteria. The enhanced antibacterial activity of the PITMS particles is probably attributed to the isothiouronium terminal amino functional group favors binding to peptide terminating with Ac-D-Ala-D-Ala in a bacterial cell-wall [16]. NPs have a larger surface-to-volume ratio compared to microparticles, hence more antibacterial functional groups are on the surface of each particle [41]. This could explain the improved antibacterial activity of the PITMS NPs compared to the microparticles.

Antibacterial Activity of the PMSF NPs

The antibacterial activity of the PMSF NPs was evaluated by determining the MIC values for all the bacterial strains tested. Each well contained 10^5 CFU/mL of each bacteria. The stock solution of the PMSF NPs was diluted in two-fold serial dilutions ranging from a concentration of 1 to 0.008% in NB medium. Water was used as a negative control. The bacterial growth was monitored via absorbance measurements at OD595 taken with a microplate reader (Synergy 2, BioTek instruments). All experiments were conducted in duplicates at least three independent times.

The MIC of the PMSF NPs was found to be 0.09% for E. coli and P. aeruginosa and 0.06% for *Listeria and S. aureus* (Table 1). These results indicate that the PMSF NPs

have a broad antibacterial potential against both Gram-negative and Gram-positive. The antibacterial activity of the NPs is attributed to the interaction of the positively charged quaternary ammonium and the hydrophobic alkyl chain with the cell wall of the bacteria [3,19]. In addition, these results demonstrate that the NPs are slightly more active against Gram-positive bacteria than Gram-negative bacteria. This could be explained by the loosely packed outer layer of the Gram-positive bacteria, compared to the additional phospholipid bilayer membrane in the cell wall of the Gram-negative bacteria, which protects the inner membrane against antibacterial polymers [20].

Table 1: Minimum inhibitory concentration (MIC) of the nano/micro-particles.

Bacterial Type	PITMS microparticles	PITMS NPs	PMSF NPs
E. coli, P. aeruginosa	0.50%	0.25%	0.09%
Listeria, S. aureus	0.50%	0.25%	0.06%

Thin-Coating of the NPs onto PET Films

In light of the promising antibacterial results of the PITMS and PMSF NPs, the potential of the NPs to inhibit biofilm formation was studied. The antibiofilm activity was studied using polyethylene terephthalate (PET) films coated with the aqueous NPs dispersions, with a ratio of 1:1 NPs to the film former (G-9/230). PITMS NPs (4%) and PMSF NPs (2%) were dispersed in G-9/230 film former (purchased from ACTEGA Coating & Sealants (Wesel, Germany)) 4% aqueous solution (1:1 v/v). The aqueous dispersions were spread separately on a 23 μm thick PET films by a Mayer rod of 6 μm wet thickness. The

obtained PET/PITMS and PET/PMSF films were left to dry, at room temperature, overnight. PET films were not coated with the PITMS microparticles, since the coating affects the transparency of the film. Uncoated PET and PET coated only with the film former (PET/film former) were used as controls.

Antibiofilm Activity of the PET films

The antibiofilm activity of the PET films were evaluated using the Gram-positive bacteria *Listeria* ATCC 33090 as the experimental model. The bacteria were grown overnight in tryptic soy broth (TSB, DIFCO) growth medium. In order to obtain a working solution, the bacterial cells were diluted in TSB to form a solution with an OD_{595} of 0.3 (approximately corresponds to $3*10^8$ CFU/mL). A sample of the stock solution (1 mL) was placed in a 24-well plate (DE-GROOT) containing the different films (1 cm diameter). The plates were then incubated at 25 °C under gentle agitation (100 rpm) for 20 h. The films were rinsed 3 times with distilled water, in order to remove the unattached bacteria (i.e. planktonic cells), and the attached cells were scraped from the films using a cell scrapers following the treatment with 250 µl of Tris-HCl (0.1M, pH 7.2). Serial dilutions were carried out and the cells spotted onto NB agar plates, which were then incubated at 37 °C for 20 h. Cell growth was monitored and determined by a viable cell count. All experiments were conducted in duplicates at least three independent times.

The PET/PITMS films showed a significant reduction by 2 logs in the biofilm formation of *Listeria* in comparison to the controls films. The PET/PMSF films demonstrated a reduction of 2 logs in the biofilm formation of *Listeria* in comparison to a film containing only the film former and a reduction of 1 log compared to the non-coated PET film (Figure 6).

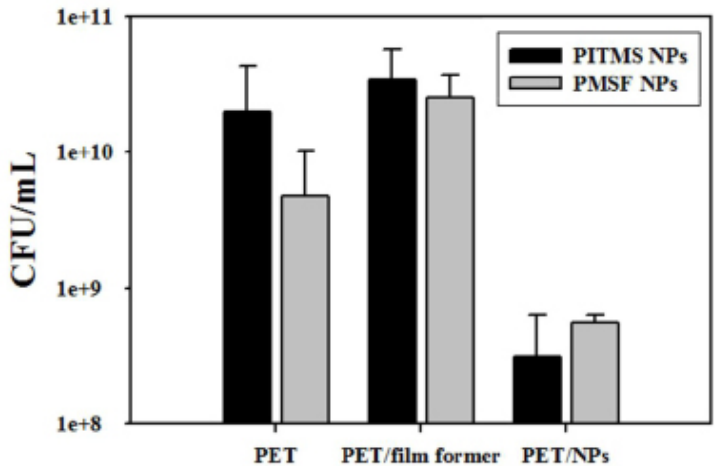

Figure 6: Biofilm formation of *Listeria* on PET, PET/film former and PET/NPs films. The data is presented as the mean ± SE.

Summary and Conclusions

This work integrates the advantages of polymer chemistry and technology with bacteriology, leading to possible developments in the formulation of new types of antibac-

terial agents. The chapter reviews the antibacterial properties of polymeric nano/micro-particles based on two monomers, MSF and ITMS, containing either quaternary ammonium or isothiouronium structures. MSF monomer has been reported to have antibacterial activity due to the quaternary ammonium functional group and the long alkyl chain [3,13]. Isothiouronium compounds and their derivatives are known to exhibit a wide range of antibacterial properties, which is attributed to the positively charged N-terminus group [9–12]. Polymeric particles, contain many functional side groups, have been shown to be less toxic than their monomers. Therefore, ITMS and MSF monomers were polymerized to form antibacterial nano/micro-particles [34,38,40].

The ITMS and MSF monomers were polymerized by dispersion co-polymerization to form antibacterial PITMS microparticles, PITMS and PMSF NPs. The nano/micro-particles have demonstrated excellent antibacterial activity against both Gram-positive and Gram-negative bacteria. These promising results encouraged the exploring of the antibiofilm properties of the NPs. Hence, PET films with a thin coating of antibacterial NPs were test against the Gram-positive bacteria *Listeria*. The PET/PITMS and PET/PMSF films exhibited a reduction in the viability of the biofilm formation of *Listeria* by 2 orders of magnitude. These results show promising antibacterial and antibiofilm properties which could be utilized for both industrial and agricultural applications.

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