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Surface modification of polyester films with polyfunctional amines: Effect on bacterial biofilm formation

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ABSTRACT

The development of materials with antifouling properties is crucial in many areas, including medicine and food packaging. In this study, 2D-matrices made of polylactic acid (PLA), polyhydroxybutyrate (PHB), or polyhydroxybutyrate-co-valerate (PHB-HV) were surface functionalized through aminolysis with three polyfunctional amines, 1,6-hexamethylenediamine (HDA), tetraethylenepentamine (TEPA), and polyallylamine hydrochloride (PAH). The aminolysis procedure was thoroughly studied to ensure a high amount of amine groups while preserving the structural properties of the films. Interestingly, PHB and PHB-HV were found to be more sensitive to aminolysis than PLA, and the highest amino group density was achieved in surfaces etched with PAH. A decrease in the contact angle from ca. 85° to ca. 70° was revealed for polymers functionalized with HDA and TEPA and a drastic reduction in *Staphylococcus epidermidis* adhesion was observed for PHB-HV functionalized with the polymeric amine PAH. Polymer antimicrobial activity was found to be related to the degree of surface functionalization. The functionalized, cationic polymer surfaces were supposed to act upon contact with bacteria, without releasing any antimicrobial agent. The developed bioactive surfaces may have potential applications as flexible films for food packaging.

1. Introduction

Biofilm formation due to bacteria adhesion onto surfaces is a major concern across various fields, such as biomedical, food industry, and agriculture [1]. Biofilms can cause antibiotic-resistant infections [2], affect food processing [3], and lead to plant bacterial wilt [4]. Bacterial adhesion involves the use of flagella and pili, and it becomes irreversible due to the production of exopolysaccharides and other adhesive components [5]. Preventing the adhesion and proliferation of pathogens is key to controlling biofilm formation [6]. Antimicrobial surfaces were traditionally obtained by adsorption or conjugation of drugs [7–10]. However, in recent years, natural and synthetic polymers with intrinsic antimicrobial and antifouling properties have been investigated for their potential [11,12]. These include PEG grafting or coating [13–15], host defense peptide-mimicking polymers [16], phenol-functionalized polymers [17] and cationic polymers [18–22].

Cationic polymers, containing positively charged amino functional groups, have shown promise as effective antibacterial agents. They do not cause drug resistance, as they kill bacteria by damaging their cell membrane. These polymers can interact with the negatively charged bacterial membranes, leading to permeabilization and lysis [23,24]. Cationic polymers have exhibited killing activity against several bacterial species and viruses, including SARS-CoV-2 [25–27]. However, the use of cationic polymers is not without limitations, and their effectiveness may vary depending on the bacterial strain and the polymer's physicochemical properties. Nonetheless, cationic polymers hold great potential in preventing biofilm formation and controlling bacterial infections.

In recent years, biodegradable and bio-based polymers have emerged as sustainable alternatives to traditional oil-based plastics. Bioplastics global production is expected to increase around 2.43 million tonnes in 2024 [28,29].

Polyesters like polylactic acid (PLA) and polyhydroxyalkanoates (PHAs) are emerging bioplastics that are made from renewable sources and are contributing to a more sustainable plastic life cycle as part of the circular economy. These polymers and their copolymers are commonly

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used in packaging [30] and biomedical applications [31] due to their biocompatibility and biodegradability. However, their lack of functional groups on the polymer backbone and low hydrophilicity hinder their interactions with molecules, including drugs.

Various methods have been explored to functionalize polyester surfaces, such as surface coating [32], layer-by-layer assembly [33], plasma treatment [34], hydrolysis [35] and aminolysis [36]. Aminolysis is a reaction between primary amines and esters that does not require a specific apparatus and has a known mechanism. Moreover, the density of the functional groups can be adjusted by changing the reaction conditions and the type of amine used [37]. PLA has been successfully aminolyzed using ethylenediamine [37], taurine [38], polyallylamine [39], and choline taurinate ionic liquid [40]. Polycaprolactone [41,42] and polyethylenterephthalate [43,44] have also been aminolyzed. However, there is limited research on PHB in this regard, and no studies have been conducted on bacterial biofilm formation on either PLA or PHB aminolyzed surfaces. Therefore, it is essential to investigate the potential of aminolysis to enhance the antimicrobial properties of PHBs and its impact on bacterial biofilm formation.

In this study, the objective was to enhance the functionality of PLA, PHB, and PHB-HV copolymer through aminolysis using three polyfunctional amines: hexamethylenediamine, tetraethylenepentamine, and polyallylamine hydrochloride. These amines have varying numbers of amino groups, and their impact on the aminolysis reaction yield, NH_2 surface density, and the effectiveness of functionalized surfaces in preventing bacterial adhesion and biofilm formation was studied. The aminolyzed surfaces were subjected to physicochemical characterization, and their ability to counteract the formation of bacterial biofilms was assessed using *Staphylococcus epidermidis* as a model microorganism.

2. Experimental section

2.1. Materials

PLA (M_w = 260.000 g/mol), Polyallylamine hydrochloride (PAH) (M_w = 50.000 g/mol) 1,6-hexamethylenediamine (HDA), tetraethylenepentamine (TEPA), ethanol (EtOH), methanol (MeOH) and chloroform ($CHCl_3$) were bought from Sigma-Aldrich. PHB was provided by Biomer while Polyhydroxybutyrate-co-valerate (PHB-HV, HV 3%) by Tianan. All the reagents were used without further purification.

2.2. Polymer films preparation and functionalization

2.2.1. Preparation of polymer films

PLA was solubilised in 10 mL of $CHCl_3$ to a 5% w/V final concentration, sonicated for 20 min a 50 °C and left under stirring (500 rpm) for 15 min for complete dissolution. PHB and PHB-HV were dissolved in 10 mL $CHCl_3$ to a 3% w/V final concentration, sonicated for 1 h at 40 °C, and left under stirring for 3 h at room temperature. Afterwards, the obtained solutions were poured into glass petri dishes (∅= 5 cm) and covered with aluminium foil to let the solvent evaporate slowly. Films thickness was measured on three different areas using a digital calibre and the average values were reported.

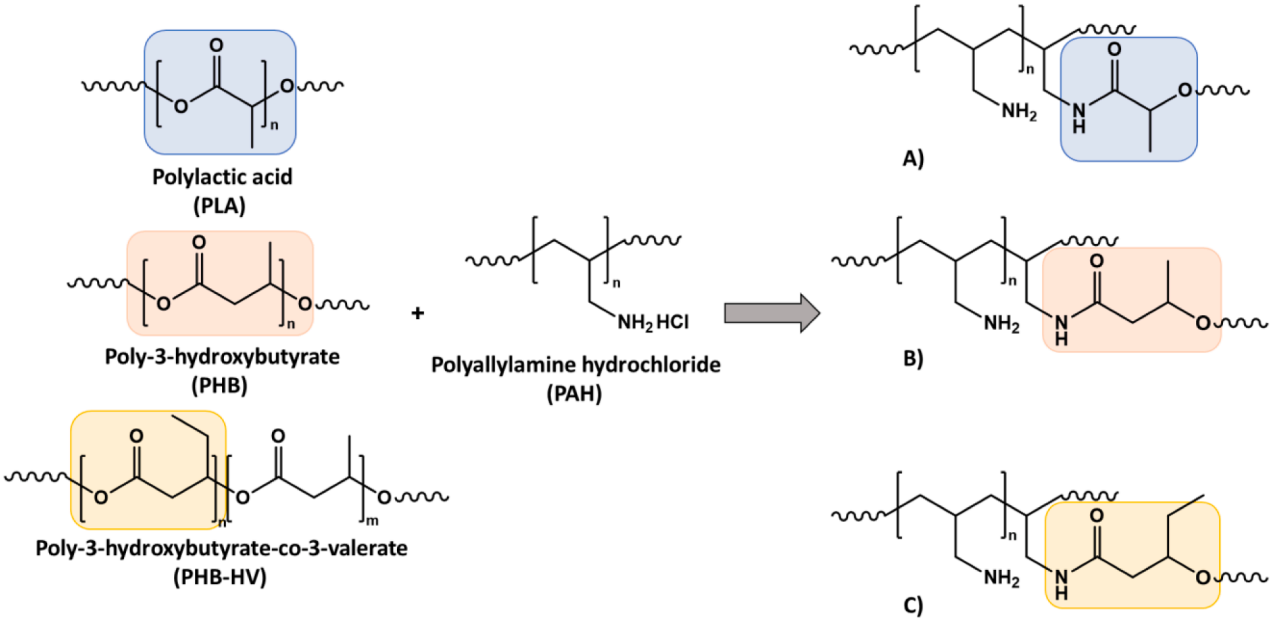
2.2.2. Aminolysis reaction on polymer films

PLA, PHB and PHB-HV were aminolyzed with either HDA, TEPA or PAH, according to the Scheme 1, in which, as an example, the aminolysis reaction is reported for the polymeric amine PAH.

The aminolysis experimental conditions were adapted from previous works [37,38]. The influence of the experimental conditions on the degree of functionalization was studied by using HDA as the model amine. Specifically, pieces of polymer films (4 mm x 8 mm) were immersed at a defined temperature (25 °C or 50 °C) for 15 min in HDA solution (5 mL) at 5, 15 or 30% w/V concentration in either EtOH or MeOH. Following the reaction, films were washed several times with distilled water, to remove the unreacted amine, until neutrality of the washing solutions and dried in vacuum oven at room temperature for 3 h.

As for TEPA, the aminolysis was carried out only at room temperature and in methanol because these conditions were found to be suitable for aminolysis with HDA.

As for the polymeric compound PAH, this amine was dissolved in water at either 5% or 15% w/V. Then, polymer films were put in contact with PAH solution and the reaction was carried out in water at 60 °C for 60 min in presence of few drops of 0.1 M NaOH to neutralize the PAH-HCl salt. Afterwards, films were washed with DI water to remove unreacted PAH. The obtained films were stored in a desiccator at room temperature and named Polymer_Amine_X where X is the amine concentration used for aminolysis.



Scheme 1. Aminolysis reaction of PLA, PHB and PHB-HV with the polymeric amine PAH.

2.3. Characterization of polymer films

2.3.1. Fourier Transformed Infrared Spectroscopy (FTIR-ATR)

Infrared spectroscopy was employed to evaluate the success of polymer functionalization. FTIR spectra were acquired in attenuated total reflection mode (ATR) using a Nicolet 6700 (Thermo Fisher Scientific, Waltham, MA, USA) with a Golden Gate Single Reflection ATR System model equipped with a synthetic diamond having an angle of incidence equal to 45°. OMNIC software was used to process data obtained during the experiment. Measurements were conducted in a spectral range within 4000–650 cm^{-1}, with a resolution of 4 cm^{-1} and 200 scans per spectrum.

2.3.2. Gel permeation chromatography (GPC)

The weight average molecular weight (M_w), number average molecular weight (M_n) and polydispersity index (PDI = Mw/Mn) of PHB-HV samples were analysed by GPC using a chromatograph equipped with a pump (JASCO PU-4180), a guard column and two columns in series (TSKgel®G6000-HHR TSKGel GMH_{HR}-H), a column oven (JASCO CO-4060) and a refractive index detector (JASCO RI-4030). Chloroform was used as eluent at a flow rate of 1 ml/min. The detector was at 35 °C, whereas the columns were at 40 °C. Polystyrene standards (0.013–3.05 × 10^6 g/mol) were used to calibrate the system.

Control PHB-HV and the aminolyzed PHB-HV_HDA and PHB-HV_PAH samples were dissolved in chloroform at a concentration of 0.3% wt/v and filtered with WHATMAN 0.2 μm PTFE syringe filters.

2.3.3. Nuclear Magnetic Spectroscopy (NMR)

^1H and ^{13}C NMR spectra were recorded on a Bruker AVANCE-400, operating at 400.13 MHz for the proton and 100.61 MHz for the carbon spectra. 1024 and 16,384 number of scans were chosen for proton and carbon spectra, respectively. NMR tubes were prepared by dissolving ca. 10 mg/mL of sample in $CDCl_3$ (7.26 ppm).

2.3.4. Ninhydrin colorimetric test

Effectiveness of the aminolysis was confirmed by determining the concentration of free amino groups on the polymer surface using the ninhydrin staining [45]. Indeed, amines react with ninhydrin producing a blue compound (Ruhemann's purple), which absorbs at 570 nm.

Aminolyzed polymer pieces (4 mg) were immersed in 2 mL of ninhydrin in methanol at a 0.2% w/V concentration. Ninhydrin was left reacting at 100 °C for 20 min and then cooled down at room temperature. Then, the absorbance of the solution was measured at 570 nm to determine the moles of amine present on the polymer film by using the calibration curves previously obtained for each amine. Six to eight standard dilutions in an appropriate concentration range for each amine were prepared for the calibration curve. Data were expressed as mole of amine per gram of sample or per surface unit of sample.

2.3.5. Elemental analysis

C, N, H and S contents were conducted by EA 1110 CHNS-O instrument to evaluate nitrogen percentage (N%) after aminolysis reaction.

2.3.6. Static contact angle

To determine films wettability before and after aminolysis, static contact angle was measured at room temperature by the drop method. A water droplet (Milli-Q-water, 10 μL) was deposited on each film surface, and pictures were taken. Images were elaborated with the Motic Images Plus 2.0 to define the base length (D) and height (h) of the drop. The contact angle (θ) was determined as follows:

$$\theta = 2\,\mathrm{arctg}\frac{2h}{d}.$$

The reported contact angles were the average values of three measurements.

2.3.7. Field Emission Scanning Electron Microscopy (FESEM)

Films' morphology was observed using high resolution FESEM (AURIGA Zeiss). Before the analysis, each film was sputtered with gold.

2.3.8. Differential Scanning Calorimetry (DSC)

Thermal properties of the films before and after aminolysis were studied by DSC by using a METTLER TA 3000 calorimeter equipped with a TC 10A processor.

Analyses were performed on 5 mg of polymer films in the 0 ÷ 200 °C temperature range at a 10 °C/min heating rate and under Nitrogen. Two cycles of heating were performed when needed. Glass transition (T_g), melting (T_m), crystallization (T_c) temperatures were determined using the SoftwareStar Evaluation.

Crystallinity percentage (χ_c) of the materials was calculated according to the following equation:

$$\chi_c(\%) = \frac{\Delta Hm}{\Delta H_m^{\circ}} \times 100$$

where, ΔH_m(J/g) is the melting enthalpy and ΔH_m°(J/g) is the theoretical melting enthalpy of the 100% crystalline polymer. $\Delta H_m^{\circ}(PLA) = 93.7\,J/g$, $\Delta H_m^{\circ}(PHB \text{ and } PHB\text{-}HV) = 146\,J/g$.

2.3.9. Mechanical measurements

Mechanical features of films, like Tensile Strength (TS), Young's Modulus (E), Elongation at break (") and Tenacity (T), were determined by stress-strain measurements by using an ISTRON 4502 (Instron Inc., Norwood, MA, USA). For the test, films were cut into rectangular specimens (3 cm × 0.5 cm × 0.1 mm) and fixed to the two clamps. The strain rate was set at 1 mm/min and a 2 kN loading cell was used.

2.3.10. Antimicrobial activity

Microbial biofilm formation on aminolyzed polymers was evaluated using *Staphylococcus epidermidis* (ATCC 35984) as the model microorganism. Firstly, a microbial suspension grown overnight in Muller Hinton broth (MH) was diluted in glucose-added MH medium (0.1% w/V) to a concentration of 1 × 10^8 CFU/mL (OD_{625} = 0.1). Then, the microbial suspension was diluted to obtain a final concentration of 1 × 10^7 CFU/m and aliquots (500 μL) were placed into 24-well plates containing each polymeric films (0.5 cm x 0.5 cm). Finally, MH broth (500 μL) was added to reach a final microbial concentration of 5 × 10^6 CFU/mL. After overnight incubation at 37 °C, the suspension was removed from the wells and films were washed twice with 0.1 M phosphate buffer (PBS) to remove loosely adherent bacterial cells. The ultrastructural analysis of biofilm grown on the polymeric surfaces was performed by FESEM. Particularly, samples were fixed with a glutaraldehyde solution (2.5% w/V) at room temperature for 30 min. Later, samples were dehydrated and dried by immersion in EtOH gradients and hexamethyldisilazane for 10 min. In the end, each treated film was fixed on the sample holder, gold sputtered and observed by FESEM.

2.3.11. Cell cytotoxicity assessment

Human dermal primary fibroblasts obtained from adult male patients [46], were used. Cells were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine, 1% Na-pyruvate and 1% non-essential amino acids (Sigma-Aldrich, Co. Saint Louis, MO, USA). To assess a potential toxic effect of PHB-HV and PHB-HV_PAH_15 films on fibroblast cells an MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium)-based colorimetric assay was performed (Promega Corporation, Madison, WI, USA). Briefly, 8 × 10^3 cells per well were seeded on films in a 96-well plate and left grown for 72 h. Cells seeded on cell culture wells were used as control (CTL). After 72 h of culture, 100 μL MTS solution was added to the wells and spectrophotometric absorbance was directly measured at 492 nm after 3

h incubation.

2.4. Statistics

Analysis of variance comparisons was performed using Mini-Tab. Differences were considered significant for $p < 0.05$. Data are reported as means ± SD.

3. Results and discussions

The surface chemical composition is a critical feature in determining the performances of a material. In the biomedical field, the presence of functional groups can largely affect the interactions of the material with cellular components thus influencing the host defense response. Functional groups can also permit to tune the interaction with specific compounds, including drugs and biomolecules. Polyesters, PLA, poly (glycolic acid) (PGA), poly(e-caprolactone) (PCL) and PHB are widely studied for a number of different applications, especially in the packaging and biomedical sectors. These materials are biocompatible and biodegradable but suffer from a lack of functional groups in the polymer backbone and side chain.

In the biomedical field, the presence of functional groups can significantly impact the interactions of the material with cellular components, thereby influencing the host defense response. Additionally, functional groups can be used to tune the interaction of the material with specific compounds, including drugs and biomolecules.

To address this issue, aminolysis, a surface modification method that introduces amino groups into polyester surface, was carried out using three amines (HDA, TEPA, and PAH) in this study. The goal of the aminolysis reaction was to etch the ester bonds in the backbone and introduce amino groups in the polyester side chains. The reaction was carried out on the surface of polymer films, where the folded chain regions were expected to be preferentially etched (Scheme 2).

During aminolysis, *single grafting* is desirable as it allows free amino groups to remain on the surface chains. *Multipoint grafting* is also possible but has a negative impact on the density of amino groups.

3.1. Influence of aminolysis reaction conditions on PLA aminolysis with HDA

The study began by investigating the effect of aminolysis reaction conditions on PLA functionalization using HDA as the model amine. The goal was to identify conditions that would allow for a satisfactory degree of surface functionalization without compromising the material's physico-mechanical properties. Achieving a good amino group density on the film surface is crucial, but it's equally important to maintain the film's structural integrity. As a result, the aminolysis reaction should primarily involve etching the polymer chains on the film surface, with the bulk properties only marginally affected. In general, interfacial aminolysis of polyesters leads to surface erosion, which increases with reaction time up to a plateau corresponding to the consumption of the surface ester bonds [47].

In Fig. 1A, the FTIR spectra of PLA films before and after aminolysis with HDA at 50 °C in ethanol at variable amine concentrations are reported. The FTIR spectrum of PLA (Fig. 1A) reveals various peaks related to its stretching and bending modes, such as the peaks at around 2900 cm^{-1} for CH_2 and CH_3 stretching, 1750 cm^{-1} for C=O stretching, 1450 cm^{-1} for CH_3 bending, and 1180 cm^{-1} for C-O-C stretching. Upon aminolysis, new characteristic peaks appear in the spectra of the films, such as the peak at 1640 cm^{-1} for CO-NH stretching, 1540 cm^{-1} for N-H bending, and 3300 cm^{-1} for N-H stretching, confirming the success of the aminolysis reaction. As expected, the intensity of these peaks significantly increased with the increase in the amine concentration (Fig. 1A). However, a high degree of functionalization resulted in extremely brittle and opaque films.

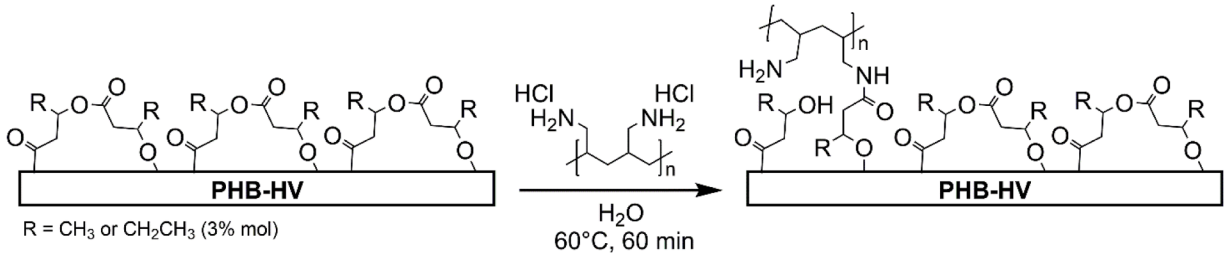
To tune the functionalization degree, the reaction was carried out at room temperature with the lowest amine concentrations (5% and 15% w/V) in both ethanol and methanol. The adsorption at 1640 cm^{-1} was much less intense, indicating a lower extent of aminolysis at room temperature (Fig. 1B). Methanol was found to be a better solvent for the reaction, as evidenced by the ratio between the intensity of the peak at 1640 cm^{-1} and the peak at 1180 cm^{-1} (Supporting info, Table S1). PLA aminolysis was promoted by an increase in amine concentration from 5% to 15% w/V when using methanol (Supporting info, Table S1).

Therefore, methanol and room temperature were selected as the best conditions and were applied for aminolysis with PHB and PHB-HV with HDA and TEPA. However, the conditions were too mild for PLA functionalization with the polymeric amine PAH, and hence the reaction was carried out at 60 °C in NaOH 0.1 N in this case.

3.2. Assessment of the extent of the aminolysis reaction on PLA, PHB and PHB-HV

Fig. 2 presents the FTIR ratio of the intensity of the peaks at 1640 cm^{-1} and at 1180 cm^{-1} for PLA at 5 and 15% amine concentration (Fig. 2A) and for all three polymers at 15% amine concentration (Fig. 2B). Supporting Info (Fig. S1) shows the FTIR spectra of the PHB-HV films aminolysed with HDA and PAH, as an example. Results showed that PLA's degree of functionalization increased with an increase in amine concentration (Fig. 2A). At 15% amine concentration, the level of amide groups introduced by aminolysis was similar for all three amines. However, the density of surface amino groups is expected to differ due to the different functionalities of the amines used (bifunctional HDA, penta-functional TEPA and polyfunctional PAH), which could affect the surfaces' antifouling properties. The study also found that PHB and PHB-HV copolymers were more reactive to aminolysis than PLA, as evidenced by a generally greater $A_{CO\text{-}NH}/A_{C\text{-}O\text{-}C}$ ratio for these matrices (Fig. 2B).

The literature suggests that the reactivity of polyesters varies due to several factors, including surface wettability, surface density of ester bonds, crystallinity, and morphology of the polyester substrate [36]. In this study, the reactivity of polyesters followed the order of PHB-HV > PHB > PLA. Even though PLA had a higher surface density of ester groups than PHB and PHB-HV, its compactly packed crystalline structure may have hindered the contact between amines and ester bonds. A similar hypothesis was done to justify the PLA aminolysis rate slower than PCL [48]. As it will be later on described, SEM observations indicated that PHB and PHB-HV had a rougher surface than PLA, resulting in



Scheme 2. Reaction scheme of PHB-HV films surface aminolyzed with PAH.

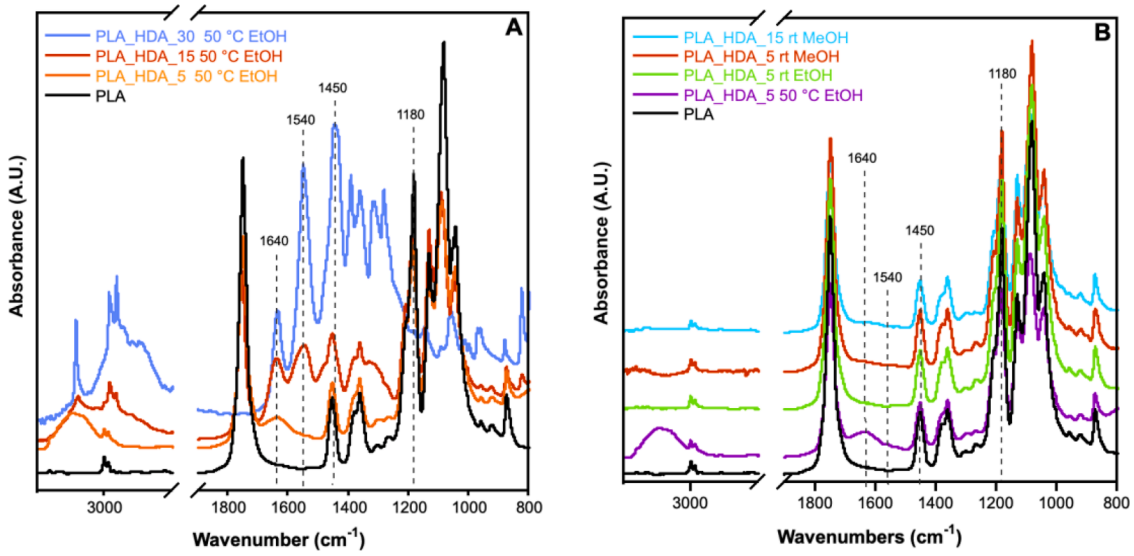


Fig. 1. FTIR spectra of PLA aminolyzed at 50 °C in ethanol with HDA at 5%, 15% and 30% w/V amine concentration (A) and PLA aminolyzed at room temperature in ethanol or methanol with HDA at 5% or 15% w/V concentration (B). Lines at 1640 cm^{-1} and 1540 cm^{-1} are related CO-NH amide adsorptions while lines at 1450 cm^{-1} and 1180 cm^{-1} are reference adsorptions.

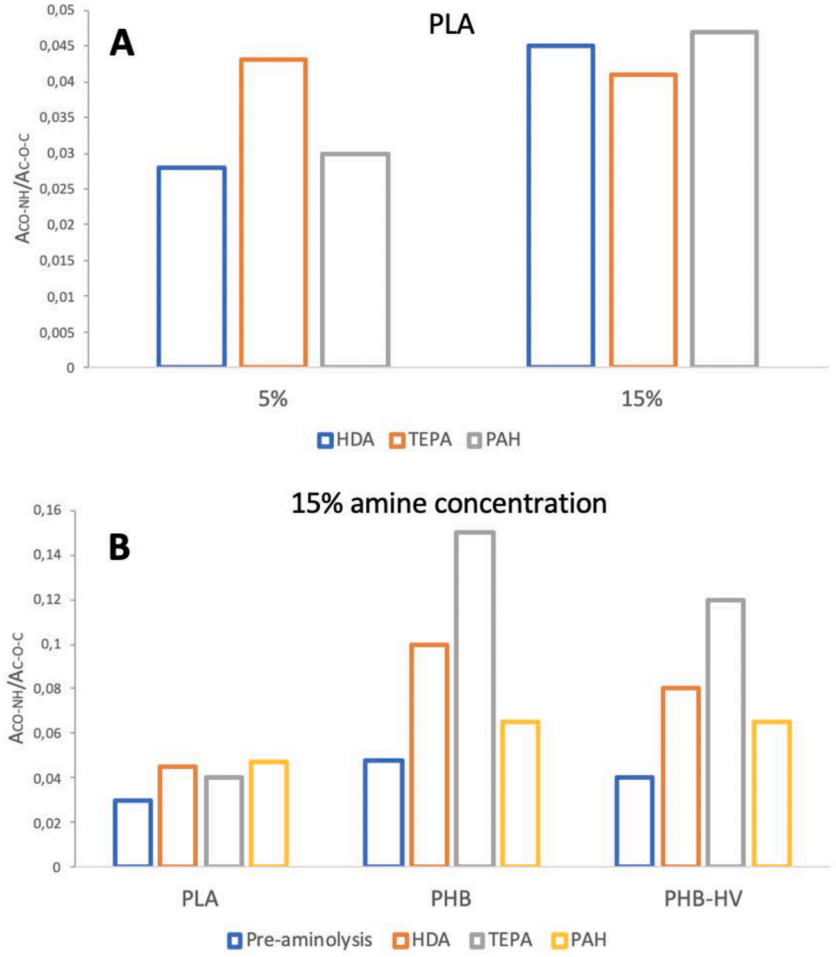


Fig. 2. ATR-FTIR ratio between the intensity of the peak at 1640 cm^{-1} (stretching CO-NH) and the peak at 1180 cm^{-1} (stretching C-O-C) for PLA aminolyzed with HDA, TEPA and PAH at 5 and 15% amine concentration (A) and for PLA, PHB and PHB-HV aminolyzed with HDA, TEPA and PAH at 15% concentration (B).

a higher surface area that could be beneficial for aminolysis.

^{1}H-NMR and ^{13}C-NMR spectroscopy were also performed to detect the presence of amide groups in both the pure and aminolyzed films. Supporting Info includes the ^{1}H-NMR and ^{13}C-NMR spectra of PHB-HV before and after aminolysis using HDA and PAH (Figs. S2–S4) as an example. No significant changes were observed in the spectra post-aminolysis, which could be because the number of introduced amide groups in relation to the polymer repeat units is below the detection limit of these techniques.

The gel permeation chromatography was conducted exclusively on PHB-HV samples, which demonstrated the most promising antifouling activity, as will be discussed later. The results revealed a noticeable alteration in the molecular weight and polydispersity index (PDI) of the polymer, confirming its etching through aminolysis. Table 1 presents the number average molecular weight (M_n), weight average molecular weight (M_w), and PDI values for PHB-HV before and after aminolysis with HDA and PAH at a 15% amine concentration.

Since aminolysis is a type of polymer degradation caused by the cleavage of ester bonds, it can lead to a decrease in M_n and M_w. In the case of PHB-HV film aminolyzed with HDA, a reduction in both M_n and M_w was observed. Similar effects were seen in the aminolysis of PCL films with 5% HDA at 30 °C for 30 min, resulting in an 11.6% decrease in M_w [47]. PLA microspheres aminolyzed with a 6% HDA/n-propanol solution at 60 °C for 60 min also demonstrated mass loss of 30% [49].

A different behavior was observed for the PHB-HV_PAH_15 aminolyzed film, which exhibited a higher M_w and PDI compared to pure PHB-HV. This result can be attributed to the use of a polymeric amine, PAH with a 50 kDa M_w, during the polymer aminolysis process. The decrease in molecular weight due to chain cutting was compensated by an increase in molecular weight resulting from amine binding. The GPC chromatograms (GPC) showed only one peak in all cases, with its shift being a consequence of aminolysis. These findings are presented in Supporting Info, Fig. S5.

The presence of nitrogen in the functionalized polymer surfaces was confirmed through elemental analysis, as shown in Table S2 of the Supporting Information. To assess the density of surface amino groups for the different polymers, a colorimetric test was conducted using ninhydrin. Although ninhydrin does not absorb in the visible region, it reacts with primary or secondary amines to form a vivid blue-colored compound with absorptions at 405 nm, 490 nm, and 570 nm. We used the absorption at 570 nm to determine the amine group concentration.

Fig. 3 displays the density of surface amino groups for the aminolyzed surfaces at a 15% amine concentration, expressed as μmol of NH_2 groups per mm^2. Notably, aminolysis with HDA resulted in a lower density of surface amino groups, which is likely due to the fact that HDA is a bi-functional compound. In contrast, the polymer surfaces etched with the polymeric amine PAH consistently exhibited the highest amino group density across all systems. These findings highlight the importance of considering the type of amine used during aminolysis, as it can significantly impact the resulting density of surface amino groups.

3.3. Physico-chemical characterization of aminolyzed PLA, PHB and PHB-HV

3.3.1. Contact angle

The introduction of amino and hydroxyl groups on the surfaces of polymers can significantly alter their surface wettability, which is a crucial factor in determining their performance in various applications. Static contact angle measurements were used, as shown in Fig. 4, to assess the surface wettability of aminolyzed surfaces with 15% amine concentration.

PLA, PHB and PHB-HV exhibit a contact angle of 83°–85°, which is slightly lower than the 90° threshold indicating the transition between a hydrophilic and hydrophobic surface. However, after functionalization with HDA and TEPA, a decrease in contact angle was observed, suggesting greater surface wettability of the etched surfaces. Interestingly, using the polymeric amine PAH for polymer aminolysis either did not vary or slightly increased the contact angle, possibly due to the alkyl backbone of the polymer chain suppressing the hydrophilicity of the amino groups. It is worth noting that with PAH, multipoint grafting is probable to occur together with single grafting. Multiple grafting refers to the reaction of two or more amino groups of one PAH molecule with the polymer ester bonds. This is an unwanted phenomenon as it reduces the exposure and availability of surface amino groups and increases the contribution of the hydrophobic PAH alkyl chain to the surface wettability features. Monnier et al. found a similar trend when aminolysing PLA with HDA at different times, where a decrease in surface wettability was observed with high functionalization degrees, attributed to double grafting [50].

3.3.2. SEM observations

Polymer surface morphology was significantly affected by aminolysis (Fig. 5). It has been observed that PLA has a generally smooth surface with just a nanometric roughness, while PHB displays a relatively rough surface with a regular pattern attributable to the lamellar edges of the internal spherulites. PHB-HV surfaces are also rough, with observable pores formed during the solvent evaporation process. The formation of a porous structure during solvent casting could be attributed to the condensation of water droplets on the polymer surface during solvent evaporation [51]. The structuring of the polymer at the water-solution interface is a significant factor in determining the formation of pores, influencing the stabilization of droplets at the interface, the point of precipitation, and the viscosity of the solution [51]. The porosity of the PHB-HV film may increase the surface area available for the aminolysis reaction, leading to greater surface wettability. Aminolysis with HDA significantly altered the surface morphology of PLA films, leading to the development of fractures and an increase in surface roughness. In contrast, aminolysis with TEPA and PAH had a smaller impact on the surface morphology of PLA films due to their lower degree of functionalization.

Similarly, PHB aminolysis with HDA and TEPA resulted in increased surface roughness, which was attributed to the partial removal of the amorphous fraction caused by the polymer etching by amines. This effect was also observed in PLA aminolyzed with taurine [39]. In the case of PHB-HV, aminolysis had a minimal impact on the surface morphology, with only a slight increase in film porosity observed, particularly with HDA and TEPA. It is worth noting that surface roughness and porosity can significantly impact the aminolysis reaction and surface wettability. In general, the aminolyzed samples didn't show any macroscopic fractures, except for PLA_HDA_15, and, therefore, they seem to be promising under an applicative point of view.

3.3.3. DSC analysis

To investigate the effect of aminolysis on polymer crystallinity, DSC analysis was conducted on both pristine and aminolyzed polymer samples (Fig. 6). Our findings for PLA (Fig. 6A) indicate that the thermal properties remained largely unaffected, which is a positive indication that the etching process occurred primarily on the polymer chains present on the polymer surface. However, a slight decrease in the glass transition temperature (T_g) and an increase in crystallinity in the film aminolyzed with HDA were observed (Table 2). These results suggest that the aminolysis reaction was more effective when HDA was used,

Table 1
Number average molecular weight (M_n), weight average molecular weight (M_w) and polydispersity index (PDI) of PHB-HV before and after aminolysis with HDA and PAH (15% amine concentration).

SAMPLE	M_n (kDa)	M_w (kDa)	PDI
PHB-HV	254	547	2.15
PHB-HV_HDA_15	147	392	2.66
PHB-HV_ PAH_15	250	630	3.01

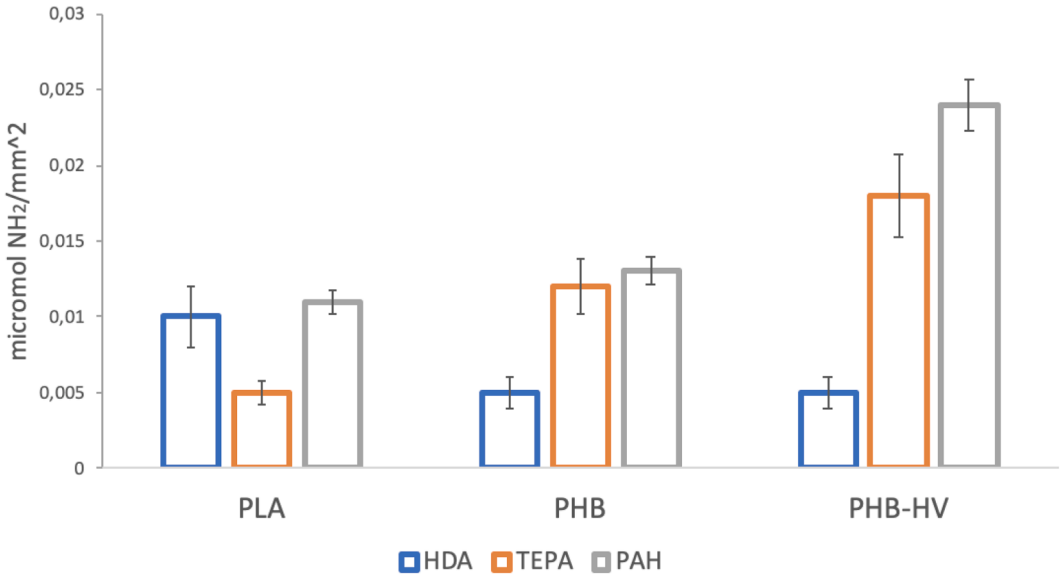


Fig. 3. Density of surface amino groups for the aminolyzed samples with 15% amine concentration, as determined by the ninhydrin colorimetric test.

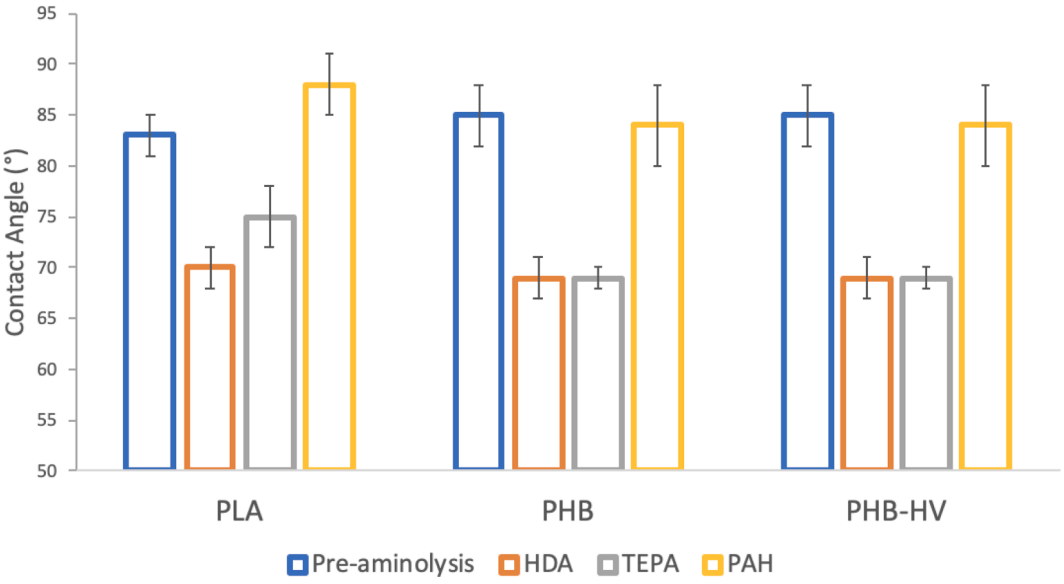


Fig. 4. Contact angle of aminolyzed polymer surfaces with 15% amine concentration.

leading to a significant increase in the extent of the reaction.

The aminolysis process, using HDA and TEPA, resulted in variations in the PHB melting peak. The PHB_HDA_15 and PHB_TEPA_15 matrices exhibited two melting peaks, centered at lower temperatures compared to pristine PHB.

The presence of two melting peaks in PHBs is a phenomenon that has been previously reported. [52,53] Upon crystallization from either melt or solution, PHB typically crystallizes in the α-form [54], which is characterized by an orthorhombic unit cell with dimensions a = 0.569 nm, b = 1.318 nm, and c = 0.589 nm. [55] The two melting peaks have been attributed to a number of factors, including melting and recrystallization processes during DSC heating, as well as the presence of different morphologies such as variations in lamellar thickness, distribution, perfection, or stability [55,56].

The total crystallinity of the aminolyzed PHB slightly increased (Table 2), although this effect was not significant for PHB_TEPA_15 and PHB_PAH_15. A similar increase was observed in PLA and polylactic caprolactone copolymer aminolyzed with HDA, which was linked to polymer etching leading to higher mobility of shorter polymer chains [38].

In contrast, in the case of PHB-HV aminolyzed samples (Table 2), the crystallinity slightly decreased compared to pristine PHB-HV. This decrease might be attributed to a higher degree of functionalization, as evidenced by previous techniques such as FTIR and ninhydrin test. Due to the more extensive aminolysis reaction, the etching might have affected not only the amorphous regions but also the crystalline structures [57].

3.3.4. *Mechanical properties*

The mechanical properties of the polymers before and after aminolysis were also evaluated by stress-strain tests (Fig. 7). Table 3 reports the values of the Young Modulus (E), Tensile Strength (TS), elongation at break (EB) and tenacity (T) of the polymers.

Interestingly, following aminolysis with a 15% amine concentration, the materials became generally harder than the pristine polymers, as suggested by the increase in Young modulus of the aminolyzed samples.

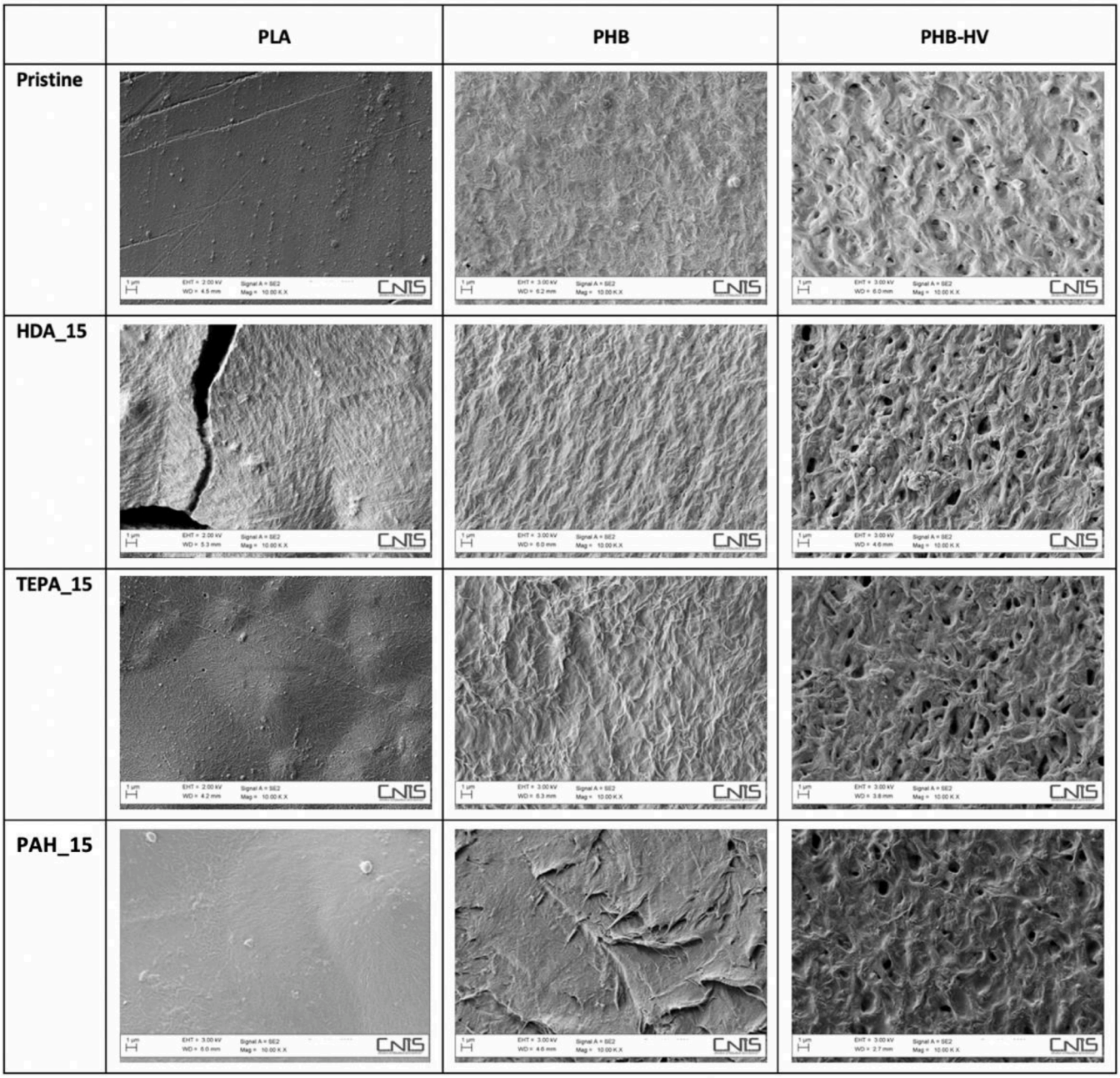


Fig. 5. FESEM micrographs of PLA, PHB and PHB-HV before and after aminolysis with HDA, TEPA and PAH at 15% concentration.

The Young modulus remained pretty much unchanged for PHB only. In addition, the samples did not lose in ductility since they showed an elongation at break similar to or even higher than the pristine polymers. Therefore, most of the aminolyzed samples showed a tenacity greater than pristine polymers (Table 3).

3.4. Evaluation of microbial adhesion and biofilm formation of polymers

The influence of positive amino groups on microbial adhesion and biofilm formation was assessed by using *S. epidermidis* as model microorganism. Specifically, the pristine and aminolyzed films were incubated for 24 h with a bacterial culture in glucose-enriched broth to promote biofilm formation. Following incubation, the microorganisms adhered on the polymer surfaces were fixed and observed by SEM. Fig. 8 shows SEM micrographs of polymers aminolyzed with HDA and PAH at 15% concentration.

The surfaces of pristine polymers are covered by a thick biofilm, in which single bacterial colonies are attached one another, forming big tridimensional aggregates. PLA functionalization with HDA and PAH did not affect biofilm formation. Indeed, the surfaces of PLA_HDA_15 and PLA_PAH_15 are completely covered by biofilm, which does not show any significant morphologic differences compared to pristine PLA. Presumably, the density of surface amino groups was not sufficient to counteract bacterial adhesion.

In contrast, a drastic reduction of bacterial adhesion can be observed on the PHB and PHB-HV surfaces aminolyzed with HDA_15 and PAH_15. Bacterial aggregates are less in number and smaller in size. Also, the biofilm surface area coverage of the aminolyzed surfaces is significantly less than pristine polymers. Among all the prepared samples, PHB-HV matrices seem to possess the best antibiofilm features since just few sporadic adherent cells are observable.

In general, bacterial adhesion onto a surface is affected by several

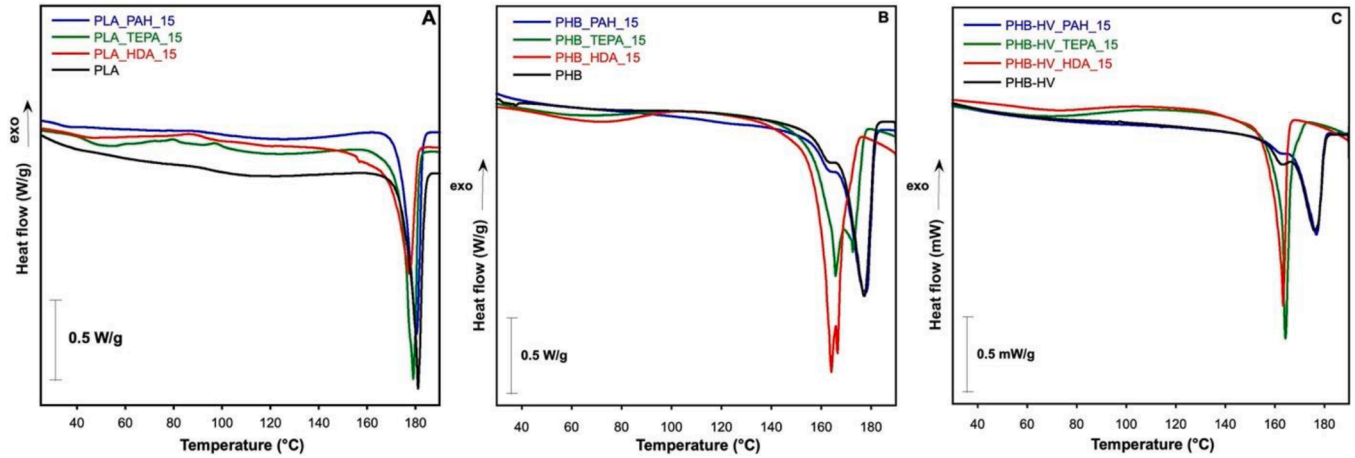


Fig. 6. DSC thermograms of PLA (A), PHB (B) and PHB-HV (C) before and after aminolysis with HDA, TEPA and PAH 15% concentration.

Table 2
Glass transition temperature (T_g), melting temperature (T_m), melting enthalpy (ΔH_m) and cristallinity percentage (χ) of polymers before and after aminolysis.

Sample	T_g (°C)	T_m (°C)	ΔH_m (J/g)	% χ
PLA	58,4	181,3	40,75	43
PLA_HDA_15	53,5	181,3	44,45	47
PLA_TEPA_15	57,1	179,2	43,41	46
PLA_PAH_15	57,2	180,5	40,40	43
PHB	–	177,3	84,1	58
PHB_HDA_15	−11	164,2	104,0	71
PHB_TEPA_15	−9	165,8	92,0	63
PHB_PAH_15	–	178,1	90,7	62
PHB-HV	–	176,3	82,7	57
PHB-HV_HDA_15	−15	163,5	79,9	55
PHB-HV_TEPA_15	−15	164,3	84,2	60
PHB-HV_PAH_15	–	176,3	75,1	51

factors including presence of specific functional groups, surface charge, wettability and morphology (roughness and topography). As far as surface wettability is concerned, superhydrophobic (contact angle higher 150°) or superhydrophilic (contact angle lower than 10°) surfaces exhibit antifouling properties [58]. Such extreme surface wettability features are usually obtained combining chemical features with hierarchical micro- and nano-structures known to amplify surface properties [58]. In our case, pristine polymers have a contact angle of 83°–85°, which decreased up to ca. 70° after functionalization with HDA and TEPA (Fig. 4). However, such decrease in wettability was not sufficient to prevent surface adhesion as evidenced by SEM observations of HDA functionalized surfaces (Fig. 8). In contrast, polymers' functionalization with the polymeric amine PAH did not change significantly surface wettability but introduced a good surface density of cationic amino groups. It is possible to hypothesize that the functionalized polymers act upon contact with bacterial cells. Indeed, cationic groups, including amino groups which are spontaneously protonated at neutral pH, can interact with the negatively charged bacterial membrane and cause membrane permeabilization and lysis. [23,24] Upon contact with the surface, bacteria are therefore killed and cannot form biofilm. Coherently with such hypothesis, polymer activity was found to be related to the degree of surface functionalization. Moreover, bacterial growth inhibition was not observed in the culture media surrounding the polymer films, suggesting that there was not release of any antimicrobial agent.

3.5. Cytotoxicity tests

In view of potential biomedical application or food contact application of the developed films, it was investigated if the aminolysis process could trigger any toxicity. Cell viability tests were performed on the film showing the best antifouling performance, e.g. PHB-HV_PAH_15, and on pristine PHB-HV. In Fig. 9, viability of fibroblast cells cultured on the two films and on cell culture-wells (CTL) for 72 h is reported.

As it can be seen, the data demonstrated lack of cytotoxicity of both films that after 72 h showed an 80% cell viability compared to control. Aminolysis of the PHB-HV surface did not affect cytocompatibility of the

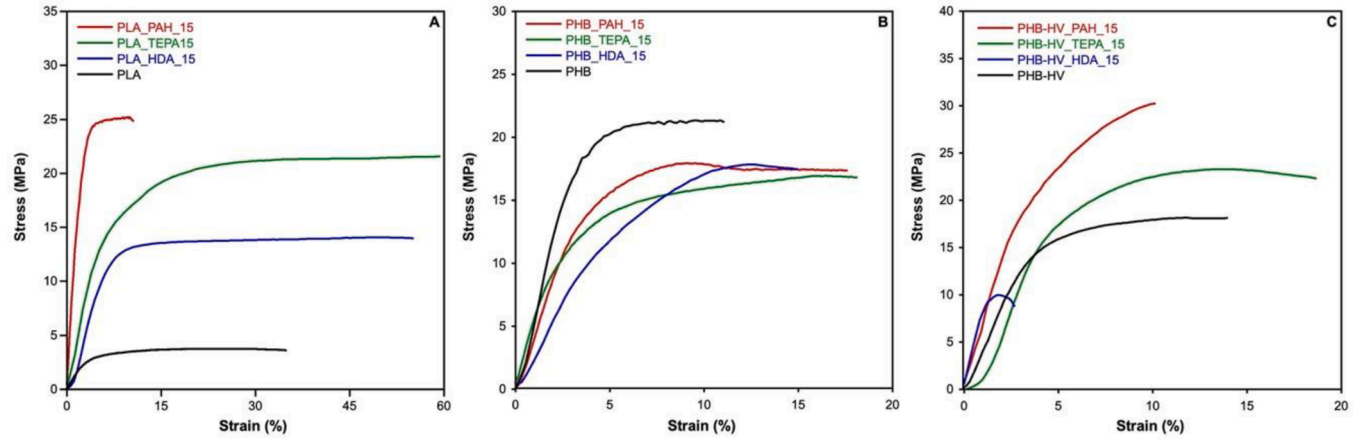


Fig. 7. Stress strain curves of PLA (A), PHB (B) and PHB-HV (C) before and after aminolysis with HDA, TEPA and PAH 15% concentration.

Table 3
Mechanical properties of polymers before and after aminolysis with the three amines at 15% concentration.

Sample	E (MPa)	Tensile Strength (MPa)	Elongation at break (%)	Tenacity (MPa)
PLA	71	3,5	35	1,2
PLA_HDA_15	183	14	55	7
PLA_TEPA_15	230	22	59	11
PLA_PAH_15	785	25	10	2,3
PHB	667	21	11	1,9
PHB_HDA_15	280	17	15	1,9
PHB_TEPA_15	500	16	18	2,6
PHB_PAH_15	465	17	17	2,6
PHB-HV	418	18	14	2
PHB-HV_HDA_15	715	9	3	0,2
PHB-HV_TEPA_15	530	22	18	3,4
PHB-HV_PAH_15	640	30	10	2,1

polymer.

4. Conclusions

In summary, this study highlights potential and limitations of aminolysis for surface functionalization of polyesters. The reaction is versatile but needs to be properly tuned to not compromise the physical properties of the polymer bulk. By changing the type of polyfunctional amine used for polymer aminolysis, it was possible to increase surface density, thus potentiating the antifouling properties of the films. Interestingly, PHB matrices were more sensitive to aminolysis and were the most efficacious in counteracting bacterial adhesion and biofilm formation. The reduction in bacterial adhesion on the PHB and PHB-HV aminolyzed surfaces was significant and, interestingly, the developed polymers are able to suppress the growth of microorganisms at the contact surface without releasing any antimicrobial agent. This aspect is particularly relevant if we consider the urgent global antimicrobial resistance issue.

The functionalization of biodegradable polyesters opens the possibility to use the obtained matrices as flexible films for bioactive food packaging. In this regard, the biodegradation behaviour of the functionalized matrices compared to pure polymers is planned to be investigated in the next future in order to strengthen their applicative potential in the packaging field.

Data availability statement

Data will be made available on request.

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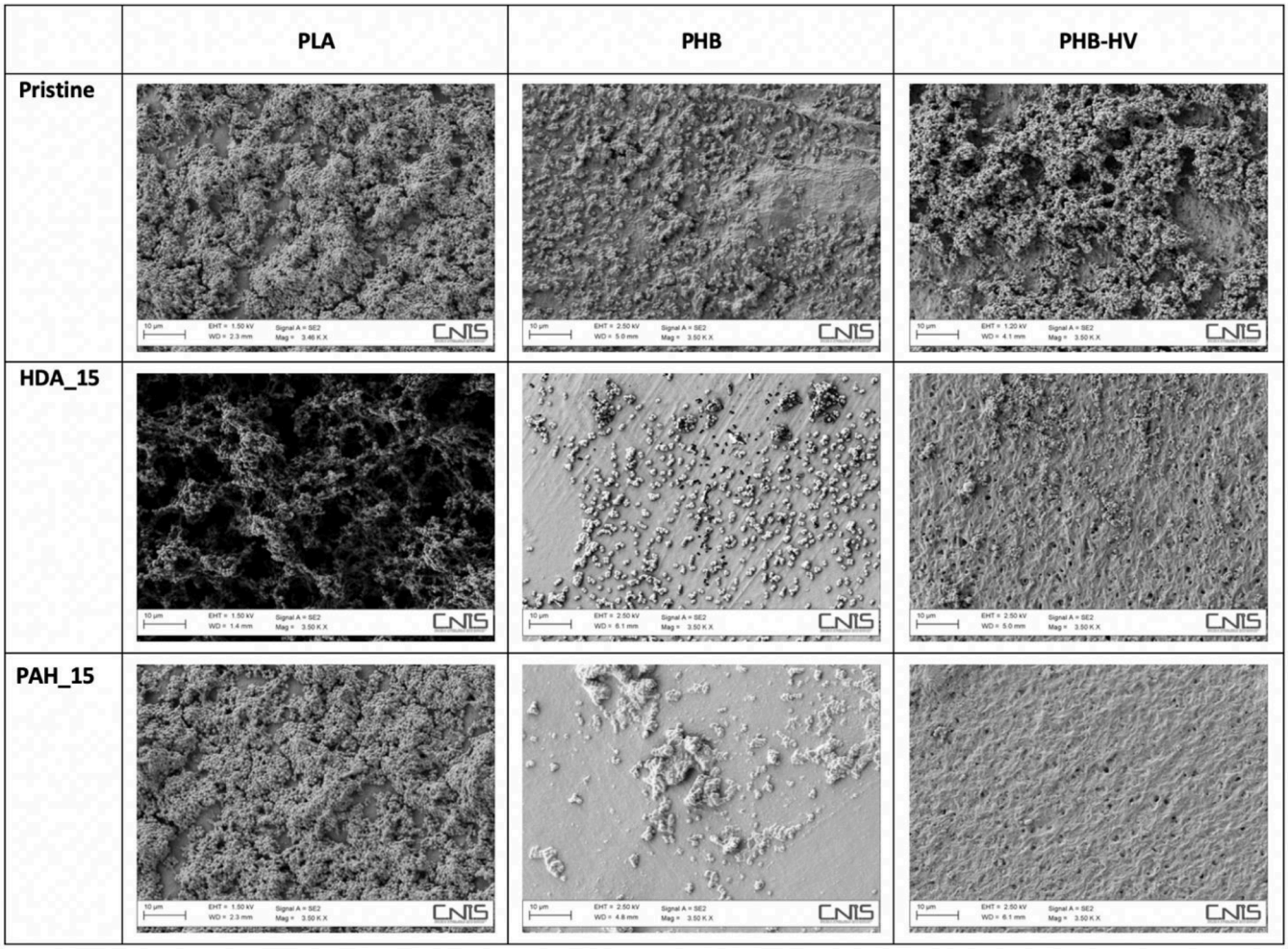

Fig. 8. SEM micrographs showing S. epidermidis biofilm on pristine polymers and aminolyzed with HDA or PAH 15% concentration. Magnification bar = 10 μm.

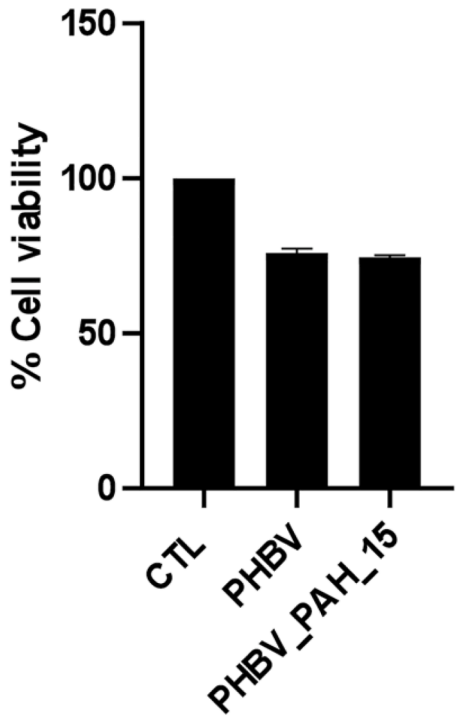

Fig. 9. Cell viability assessed by MTS colorimetric assay. Fibroblast cells were cultured on PHB-HV and PHB-HV_PAH_15 films or on cell culture-wells (CTL) for 72 h. Cell viability of cells on films was normalized to that of cells on culture-wells, which is reported as 100%.

CRediT authorship contribution statement

Gianmarco Mallamaci: Formal analysis, Investigation. **Benedetta Brugnoli:** Formal analysis, Investigation, Writing – original draft. **Alessia Mariano:** Formal analysis. **Anna Scotto d'Abusco:** Methodology. **Antonella Piozzi:** Data curation, Writing – review & editing. **Valerio Di Lisio:** Methodology, Writing – review & editing. **Elisa Sturabotti:** Methodology, Writing – review & editing. **Sara Alfano:** Methodology. **Iolanda Francolini:** Conceptualization, Data curation, Writing – original draft, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.surfin.2023.102924.

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