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Biodegradable and Bio-based Polymers

Tunable enzymatic biodegradation of poly(butylene succinate): biobased coatings and self-degradable films

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ABSTRACT

Biodegradation of polyesters driven by enzymes is considered as one of the most effective way of degradation of these materials, as a way to control plastics accumulation in the environment. In this study, we present two different strategies to tune the enzymatic degradation of PBS films triggered by a lipase from *Pseudomonas cepacia*. Firstly, the kinetics of enzymatic degradation of PBS films was regulated by applying multilayer coats of polysaccharide alginate and chitosan (Alg/Chi) films. Secondly, self-degradable PBS films were prepared by embedding lipase-filled alginate particles. For comparison purposes, a detailed enzymatic degradation study of neat PBS films exposed to a lipase from *P. cepacia* in solution was made to determine the main experimental parameters influencing their degradation in solution. The results showed that an increase in enzyme concentration increased the degradation extent and rate of neat PBS films. At a fixed enzyme concentration, stirring of the solution containing the enzyme and the PBS also increased the biodegradation rate. In the case of the PBS films coated with a different number of Alg/Chi layers by spray-assisted LbL and subjected to enzymatic degradation experiments in solution, the extent of degradation was found to be dependent on the number of protective coating layers. Therefore, the Alg/Chi biobased coating constitutes an effective barrier to the diffusion of the lipase, thus proving its effectiveness in modulating the enzymatic activity as a function of coating thickness. In the case of self-degradable PBS containing lipase-embedded alginate beads (employed to protect the enzyme during high-temperature processing), only limited biodegradation was observed as the amount of encapsulated enzyme employed was too small. Nonetheless, these results are promising, as the enzymatic activity –indicative of the degradation capacity of the enzyme– determined for all these samples was about 2 orders of magnitude lower than that of previous assays.

1. Introduction

Poly(butylene succinate) (PBS or PBSu), sometimes referred to as poly(tetramethylene succinate) (PTMS), is an aliphatic polyester that can be included in fossil-based biodegradable polymers. PBS can also be produced through biobased monomers obtained through fermentative production routes based on renewable feedstocks [1]. In soil, PBS degrades significantly faster than conventional plastics, and hence it constitutes a solution to the growing problem of accumulation of non biodegradable plastics [2,3]. Some of its potential uses include biomedical applications, packaging, and agriculture. To give a few examples, PBS has been employed to prepare polymeric mulch films to reduce plastic accumulation in soil [4], and for the fabrication of soft packaging [5]. The biomedical applications of PBS and PBS-based materials have also been evaluated recently [6]. Moreover, in a recent publication from our group, an exhaustive review of the mechanical and

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thermal properties, crystallinity, barrier properties, and the different degradation methods of PBS and PBS-based copolymers and nanocomposites was presented [7]. Despite all the possible applications of PBS, the study of its biodegradation must be conducted carefully, as it can condition its application in different fields. To that aim, the degradation studies of PBS should be in consonance with its specific applications. For example, the hydrolytic degradation could be more related to the different biomedical applications of this polyester, whereas degradation under environmental conditions (soil burial, activated sludge, compost) could be more interesting for mulching films in agricultural applications [8–10].

Most of the enzymatic degradation assays, which consist of a hydrolytic degradation catalyzed by an enzyme, are developed under physiological conditions (i.e., 37°C and pH 7-7.5) [11–13]. Enzymatic degradation is more favored for PBS samples with low molecular weight and low crystallinity [12,14], as well as at a temperature close to T_m (the PBS melting temperature is above 100°C), even though the self-stability of the enzymes at high temperature constitutes a drawback [15]. In literature, PBS enzymatic degradation presents a wide range of results, depending on the type of enzyme and experimental conditions. Some authors have reported complete PBS degradation in a few hours employing different cutinases [11,16,17] and lipases [17,18], whereas other authors observed quite low weight losses with cholesterol esterases [19]; or even negligible degradation [12,13,20]. Lipases (EC:3.1.1.3) are esterases with the ability to hydrolyze long-chain triglycerides to di- and subsequently monoglycerides [21]. Regarding the enzymatic degradation of polyesters and PBS in particular, the majority of the experiments are done with films immersed within an enzymatic solution. In addition, the encapsulation of enzymes is critical to maintain their activity, especially when high temperatures are needed. However, this step of protecting the enzymes is not straightforward, as it is important to select a good material for this purpose, and control the conditions of encapsulation.

Taking into consideration the high importance of focusing polymers (and, more specifically, polyesters) towards zero-waste materials, the biodegradation of PBS and its comprehension becomes a key point nowadays. In this paper, we report on two novel strategies to modulate the enzymatic degradation of PBS films through their combination with biobased polymers (e.g., polysaccharides) (Scheme 1). For this study, a lipase from *Pseudomonas cepacia* with a sequence composition containing 364 amino acids has been selected [22]. As a first strategy, the preparation of biobased multilayer coatings, consisting of polysaccharide polyelectrolyte complexes (PPC) of alginate and chitosan deposited onto PBS films will be assessed, and the degradation of the PBS-coated films immersed in aqueous solutions containing lipase will be monitored as a function of the time. Polysaccharide polyelectrolyte complexes (PPC) have been widely investigated as good barrier materials for paperboard and other materials conducted towards sustainable packaging applications [23–25]. On this basis, PPC have been recently incorporated into biopolyester materials as a way to improve barrier properties of these packaging materials [26,27]. In addition to this, the employment of PPC in biomedical applications is also widely reported in the literature [28,29]. In particular, alginate and chitosan have been assembled into nanostructured films using layer-by-layer (LbL) assembly to be used in several biomedical applications, such as matrixes for drug delivery modulation, and iron oxide nanoparticles encapsulation for magnetic hyperthermia therapies [30–33], due to its good stability conferred by the electrostatic interactions created among both components. However, to the best of our knowledge, the effect of PPC coatings on the biodegradation of PBS films has not been reported so far. We hypothesize that these coatings will be able to regulate the enzymatic degradation of PBS.

Although PBS enzymatic degradation successfully works under different conditions, several steps are needed to achieve it, from the fabrication of the polyester films to their biodegradation. For this reason, and as a second strategy, the preparation of self-degradable PBS triggered by enzymatic degradation is proposed, which can be a more straightforward method with respect to the PBS biodegradation carried out in solution through externally added lipases. This approach has been reported for PBS, as well as for other polyesters, such as poly(butylene succinate-*co*-butylene adipate) (PBSA) and poly(ε-caprolactone) (PCL) [34], as a novel strategy of polymer degradation through "embedded enzymatic degradation" [35]. Lipozyme *Candida antarctica* B (CALB) was employed in free form to obtain polymeric films with embedded enzymes after extrusion of both components. The weight loss reached ~18% after 500 hours, although no variation in M_w was reported, indicating that the degradation mechanism proceeded through surface erosion. For our study, we propose the encapsulation of a chosen lipase (lipase from *P. cepacia*) within alginate beads in order to prevent enzyme thermal degradation. The immobilization or embedding of enzymes has been widely studied in different polysaccharide matrices [36–38], including alginate. These lipase-embedded alginate particles have been employed for the hydrolysis of oils [39] or for the synthesis of isoamyl acetate [40]. To the best of our knowledge, the feasibility of preparing lipase-embedded alginate beads embedded in PBS films and their subsequent application in enzymatic degradation studies has not been reported yet. Such approaches could be interesting for several applications in the biomedical, agricultural or food industry fields, where degradation takes an important role. The solutions proposed in this study enable the facile modification of the degradation kinetics by employing biobased materials.

To sum up, the present work will focus on PBS enzymatic biodegradation. As a first step, the experimental conditions to carry out enzymatic degradation assays of PBS will be optimized. Then, the influence of a biobased polysaccharide multilayer coating on the enzymatic degradation of PBS will be ascertained. Finally, the preparation of self-degradable PBS-based materials will also be investigated.

2. Materials & methods

2.1. Materials

Commercial poly(1,4-butylene succinate), extended with 1,6-diisocyanatohexane (Lot #MKBX5346V and referred to as PBS, M_w ~112,000 g/mol [GPC], M_n ~81,000 g/mol [GPC], M_n ~53,000 g/mol [^{1}H-NMR]), lipase from *P. cepacia* (Lot #BCBV1950, Lot #BCCG3768, Lot #BCBP8887V), Amano lipase PS from *Burkholderia cepacia* (Lot #MKCB2061V), Lipase *P. cepacia* immobilized on Immobead 150 (Lot #BCBV3782), phosphate buffered saline (physiologic pH of 7.2-7.4), alginic acid sodium salt from brown algae of low (Lot #SLCG0203) and high (M_w ~600,000 g/mol) viscosity, chitosan (low molecular weight, Lot #BCCD9853), polyethylenimine branched (PEI), sodium acetate anhydride and Triton X-100 were purchased from Sigma-Aldrich. Calcium chloride dihydrate was acquired from Acros Organics, acetic acid glacial and 2-propanol were acquired from Scharlab and VWR, and 4-nitrophenyl palmitate (pNPP) was provided by Alfa Aesar. Sunflower oil of alimentary grade was also employed. All reagents were used as received, except chitosan, which was purified to remove impurities following a typical methodology [31].

2.2. Preparation of neat PBS hot-pressed films

Micrometric polymeric films were prepared by hot-pressing PBS pellets in a Collin 200 × 200 (Collin Solutions, GmbH, Germany) pneumatic press. The conditions for the preparation of the films consisted of four steps done at 125 °C: *i)* 5 minutes at 5 bars, *ii)* 2 minutes at 50 bars, *iii)* 2 minutes at 100 bars, and *iv)* a final extra cooling step (at room temperature) for 2 minutes at 100 bars. The average thickness of these films was 0.12 ± 0.02 mm, as determined by employing a digital caliper. The films were kept at room temperature for one week before starting the experiments.

2.3. Enzymatic degradation of neat PBS films by externally added lipases

Enzymatic degradation assays were carried out with a lipase from *P. cepacia* at two different concentrations, 2 mg/mL and 1 mg/mL. The enzymatic stock solution was prepared in phosphate buffered saline (pH 7.2-7.4). For this study, PBS films (10 × 10 mm^2) were immersed individually into 5 mL-glass vials containing 2 mL of the enzyme stock solution and placed into a thermostated chamber (Heraeus Instruments, Germany) at 37 °C with rotational stirring (Rocker 3D Digital, IKA-Werke, GmbH & Co. KG, Germany) at 45 rpm. Experiments in the absence of stirring were also carried out to evaluate the effect of this parameter on the enzyme performance.

The samples were withdrawn at certain times, washed with distilled water, and finally weighed after complete drying at room temperature (48 hours). To control the enzymatic degradation, the weight loss of the samples was determined using the following equation (Eq. (1)).

$$W_{loss}(\%) = \frac{W_{initial} - W_{final}}{W_{initial}} \cdot 100 \quad (1)$$

Where W_{loss} stands for weight loss (expressed in %), $W_{initial}$ refers to the initial weight of the samples (before degradation), and W_{final} indicates the final weight of the samples after the degradation assays.

In order to assess the performance of the lipase towards degradation, the enzymatic activity was checked before the experiment, during it, and after conducting the biodegradation. The procedure for determining the enzyme activity consisted in measuring the UV absorbance of the pNPP degradation over time at 410 nm, as shown in Figure S1 in the Supplementary Information.

The molecular weight, crystallinity, and surface morphology of the withdrawn-dried PBS films were determined to monitor changes in these parameters as a function of the degradation time. Surface morphology was observed in a Zeta-20 Optical Profiler (KLA-Tencor Corp., USA) with a 0.50x Coupler attached at different magnifications (5x, 20x, 50x, and 100x). The determination of the number average molecular weight was carried out through proton nuclear magnetic resonance (^{1}H-NMR). Spectra were recorded in a Bruker AMX-300 Spectrometer (Bruker Corp., USA). 640 scans were recorded from 10 mg sample solutions in 1 mL of deuterated chloroform. For M_n determination, signals from CH_2OH and CH_2COOH end groups were compared with the signals of COOCH_2 from samples derivatized with trifluoroacetic anhydride. Additionally, gel permeation chromatography analysis (GPC) provided information on both number and weight average molecular weights. GPC were recorded in a Waters Instrument (Waters Corp., USA) equipped with RI and UV detectors. HR5E and HR2 Waters linear Styragel columns (7.8 mm × 300 mm, pore size 10^3–10^4 Å) packed with cross-linked polystyrene and protected with a precolumn were used. Samples were prepared by dissolving 1 mg of the sample in 1 mL of chloroform and using the same solvent as the eluent. Measurements were performed at 35°C with a flow rate of 0.5 mL/min and molecular weights were calculated against monodisperse polystyrene standards. The crystallinity of the films was further evaluated by differential scanning calorimetry in a PerkinElmer DSC 8500 with an Intracooler II (PerkinElmer, Inc., USA). For DSC analysis, PBS films were weighed and placed inside aluminum pans. Temperature sweeps with two scans were done from -30 to 160 °C, at 10 and 20 °C/min.

$$X_c = \frac{\Delta H_m}{\Delta H_m^0} \quad (2)$$

The crystallinity (X_c) was determined from the melting enthalpy (ΔH_m) of the peak that appeared at 110-115 °C, which corresponds to the melting of PBS, and the equilibrium melting enthalpy (ΔH_m^0), which was considered to be 213 ± 10 J/g [41], as detailed in Eq. (2). DSC curves were analyzed with the Pyris Manager software (10.1 and 13.3 versions) from PerkinElmer, Inc.

2.4. Preparation and characterization of polyelectrolyte multilayer spray-coated PBS films

Spray-assisted layer-by-layer deposition was employed for the coating of PBS films with polysaccharide aqueous solutions following a procedure reported elsewhere [31,42]. First, PBS films were cut into 25 × 10 mm^2 and introduced individually into a polyethylenimine (PEI) solution (1 mg/mL) for 5 minutes to confer PBS a more hydrophilic surface, as well as providing a positively charged surface, for a better deposition of the polysaccharide coating [31–33,43]. After that, the films were air-dried and washed for 2 minutes with Milli-Q water. Then, they were placed onto a metallic support for the multilayer spray-coating in the automatic equipment (ND-SP Spray Coater, Nadetech Innovations, Spain). The support with the sample was set at 135° with respect to the spray jet, in order to avoid the accumulation of alginate and chitosan solutions on the sample, which would lead to the formation of heterogeneities in the coating thickness (see Scheme 2a). Three different systems with varying alginate/chitosan layers (8, 24, and 40) were prepared and designated as *PBS_nL*, where *n* stands for the number of layers. For each sample, the corresponding number of layers was deposited through a consecutive deposition of alginate (2.5 mg/mL in pH 3 buffer solution) and chitosan (1 mg/mL in pH 5 buffer solution). Both sides of the PBS film were coated consecutively. Finally, samples were left drying at room temperature for 48 hours before further experiments.

The thickness of the coating was assessed through scanning electron microscopy (SEM) employing a Hitachi SU8000 Scanning Electron Microscope (Hitachi, Ltd., Japan) and operating at 0.8 kV. Spray-coated PBS films were fractured in liquid nitrogen and gold-coated (Polaron SC7640 Sputter Coater, Quorum Technologies, Ltd., UK) prior to the SEM analysis. The values are presented as the average ± standard deviation of at least four positions in three different samples.

The surface wettability of the spray-coated PBS films (20 × 20 mm^2) and the neat PBS sample without coating was characterized by static water contact angle (WCA) using a KSV Theta Goniometer coupled with a camera (KSV Instruments, Ltd., Finland). Water droplets of 3 μL were deposited on top of the samples, and pictures were recorded at different positions. WCAs were measured with the Software KSV CAM 200 Optical Contact Angle Meter, and results are expressed as the average ± standard deviation of at least four positions in three different samples.

2.5. Nanomechanical properties of PBS and alginate/chitosan coating

As a way of evaluating the mechanical behaviour of the polysaccharide coating, nanoindentation measurements were performed using a G200 Nanoindenter (KLA-Tencor Corp., USA) with a Berkovich diamond indenter. Storage modulus (*E'*) and hardness (*H*) were determined using continuous stiffness measurements and a low load module (DCM). At least 30 indentation tests were produced at different locations to determine average values for each material (± standard deviation). For this purpose, alginate/chitosan coatings were deposited on two different substrates: PBS films and silicon wafers, both with dimensions 10 × 10 mm^2. For the sake of comparison, alginate and chitosan films were also tested. These polysaccharide films were obtained by pouring aqueous solutions of each polysaccharide (1% w/v, chitosan solution also contained 1% v/v of acetic acid) in separate Petri dishes and letting them dry in a stove set at 37 °C for 7 days.

2.6. Enzymatic degradation of PBS spray-coated films by externally added lipases

Enzymatic degradation assays were carried out with a lipase from *P. cepacia* at 2 mg/mL following the same procedure as in Section 2.3. Then, the surface morphology changes were monitored as a function of degradation time by profilometry, as mentioned in Section 2.3.

2.7. Preparation and enzymatic degradation of self-degradable PBS films

Self-degradable polyester films were prepared by hot-pressing, with an extrudate obtained from PBS pellets and polysaccharide particles with the embedded enzyme. Prior to the preparation of the films, the alginate particles with the embedded enzyme were prepared as follows. High M_w alginate was dissolved in deionized water (2% w/v) together with Amano lipase PS from *Burkholderia cepacia* (named *Amano lipase BC*). The amount of lipase inside the alginate beads was optimized by testing several concentrations of *Amano lipase BC* (0.75, 1.25, 1.75, and 2 mg/mL in 2% w/v alginate particles). Finally, the highest concentration (i.e., 2 mg/mL) was chosen for the preparation of the self-degradable PBS films, as higher concentrations of enzyme usually lead to better performance towards degradation. The beads were then fabricated using a syringe pump (Legato 200, Kd Scientific, Inc., USA) equipped with a 5 mL syringe (Inkjet Luer Solo from B.Braun, AG, Germany) and a 23 G hypodermic needle (0.6 × 25 mm 23Gx1", Nr. 16 Microlance 3 from BD, USA), by setting the flow at 800 μL/min. The alginate solution containing the enzyme was added dropwise to a 250 mL aqueous solution of calcium chloride (2% w/v). The particles, named as *AlgEmb*, were left in the $CaCl_2$ bath under stirring for at least 1 hour for hardening and then recovered and washed with distilled water. After that, the alginate beads were freeze-dried (Powerdry LL 1500, Thermo Fisher Scientific, Czech Republic), and their morphology was observed with a Hitachi TM3030 Plus Tabletop Microscope (Hitachi, Ltd., Japan). These particles are referred to as *AlgEmbLi* from now on.

In a second step, the joint extrusion of PBS and *AlgEmbLi* particles was done at a ratio of 10% w/w *AlgEmbLi* to PBS (see Scheme 2b). For the extrusion process, PBS pellets (6.3 g) and the corresponding amount of *AlgEmbLi* beads (700 mg) were pre-mixed, introduced in an MC 15 micro compounder (Xplore Instruments BV, The Netherlands) at 125 °C, mixed for 3 minutes at 60 rpm and extruded at 60 rpm. Finally, the films containing *AlgEmbLi* beads were obtained by hot-pressing at 125 °C, with a similar procedure to the one followed for the PBS films and explained in Section 2.2, employing a Polystat 100T (Schwabenthan-Maschinen GmbH & Co. KG, Germany) and a Collin P200E (Collin Solutions, GmbH, Germany) hydraulic presses.

Biodegradation assays were carried out in phosphate buffered saline solution (pH 7.2-7.4), without enzyme in this case. For this study, the previously obtained films containing *AlgEmbLi* beads were cut into 10 × 10 mm^2 sized samples, immersed individually into 5 mL-glass vials containing 2 mL of the buffer solution, and placed into a thermostated chamber (Heraeus Instruments, Germany) at 37 °C with rotational stirring (Rocker 3D Digital, IKA-Werke, GmbH & Co. KG, Germany) at 45 rpm. All the samples were weighed before starting the experiments, and the enzymatic degradation was monitored through weight loss, as indicated in the previous experiments. The samples were withdrawn at certain times, washed with distilled water, and finally weighed after a complete drying at room temperature (48 hours).

3. Results and discussion

Many works have studied the degradation of PBS within different environments, including hydrolytic, enzymatic, compost, and soil burial. Focusing on the enzymatic degradation –the media of our study–, published results from different research groups show great variability in the extent of enzymatic degradation of PBS measured through the measurement of weight loss over time, which are related to different enzymes employed, degradation conditions (there are no standard conditions, as in other degradation tests, such as soil burial or compost) or even due to differences in PBS crystallinity. This variety of conditions results in high degradation rates for cutinases [11,16,17,44], whereas lipases cover the whole range, from non-visible degradation [45] to total degradation [17,18]. The influence of the enzyme concentration has been a subject of study, as varying this parameter is one way to control and tune the degradation [46]. To that aim, herein two different strategies to modulate the enzyme-induced degradation of PBS films are investigated by tuning their performance: *i)* the fabrication of polysaccharide multilayer spray-coated PBS films (Scheme 2a), and *ii)* the preparation of self-degradable PBS films containing enzyme-embedded polysaccharide beads (Scheme 2b), and results will be compared with neat PBS films.

3.1. Effect of lipase concentration and stirring on the enzymatic degradation of PBS neat films

In a first step, the enzymatic degradation of PBS neat films was studied. For such purpose, different experimental conditions of enzyme concentration (2 and 1 mg/mL) and stirring/stationary (*stir/stat*) were employed, and samples were named as *PBS_stirring condition_lipase concentration* (e.g., films tested under stirring in a 2 mg/mL lipase solution are named as *PBS_stir_2*). Fig. 1a shows the weight loss as a function of time of PBS films immersed in lipase aqueous solutions. For the PBS films immersed in an aqueous solution of lipase at 1 mg/mL (PBS_stir_1), there is a clear tendency to achieve a lower degradation extent when compared with PBS films immersed in 2 mg/mL lipase aqueous solutions. In addition, the weight loss achieved is lower for samples immersed in the lowest concentration of lipase aqueous solution (1 mg/mL). Regarding the stirring effect, for PBS films immersed in 2 mg/mL lipase aqueous solutions, stirring seems to favor the PBS degradation slightly. An interesting effect of stirring on enzyme-degraded PBS films was related to the initial area where the degradation started. Visual observations and profilometry images revealed the lipase-induced degradation of the films subjected to stirring, starting from the central part of the film and then continuing towards the edges, showing a "hole" degradation effect (Figure S2a, in the Supplementary Information). On the other hand, PBS films tested under stationary conditions (no stirring) showed the enzymatic degradation started from the edges towards the center and the corners exhibiting a "ring" degradation effect (Figure S2b, in the Supplementary Information). Such effect could be related to the contact achieved, as it is well-known that stirring enhances the adsorption of the enzyme on the polymer surface, promoting the degradation rate [47]. However, not related examples have been found in literature regarding this effect of stirring on degradation, as some authors reported a edge-to-center degradation for PCL discs [48], whereas in a different publication, the observed degradation started from the surface rather from the edges [49]. It is important to note that the maximum weight loss of the sample used as a negative control in absence of enzyme (PBS_control) was around 1.5 wt%, which means that PBS films did not show any significant degradation under stirring at 37 °C in phosphate buffered saline solution in the absence of lipase.

The degradation kinetics, determined from the slope of the weight loss curves, showed a faster weight loss as the enzyme concentration increased (Fig. 1a). Moreover, the stirring also influences the kinetics of degradation, leading to higher weight loss values for experiments performed under stirring compared to stationary conditions. Thus, the slope of the linear fit of the data showed a value of 0.0423 %/h for PBS_stir_1, whereas PBS_stir_2 and PBS_stat_2 exhibited values of 0.0729 and 0.0567 %/h, respectively. This 70% increase in the slope value when increasing the lipase concentration from 1 to 2 mg/mL proves the importance of selecting an appropriate enzyme concentration for the enzymatic biodegradation tests.

Optical images were taken by profilometry to analyze the surface of the degraded PBS films over time. The representative results obtained for the sample PBS_stat_2 are shown in Fig. 1b. As appreciated in this figure, the surface is quite homogeneous after 48 hours (below 5% weight loss), but after 10 days, some holes are visible even if PBS films showed a moderate weight loss (~11 wt%). These results are also in accordance with previous reports, which showed the appearance of holes during biodegradation [11,17], indicating that degradation takes places through surface erosion mechanism.

It is important to note that variations of crystallinity and molecular

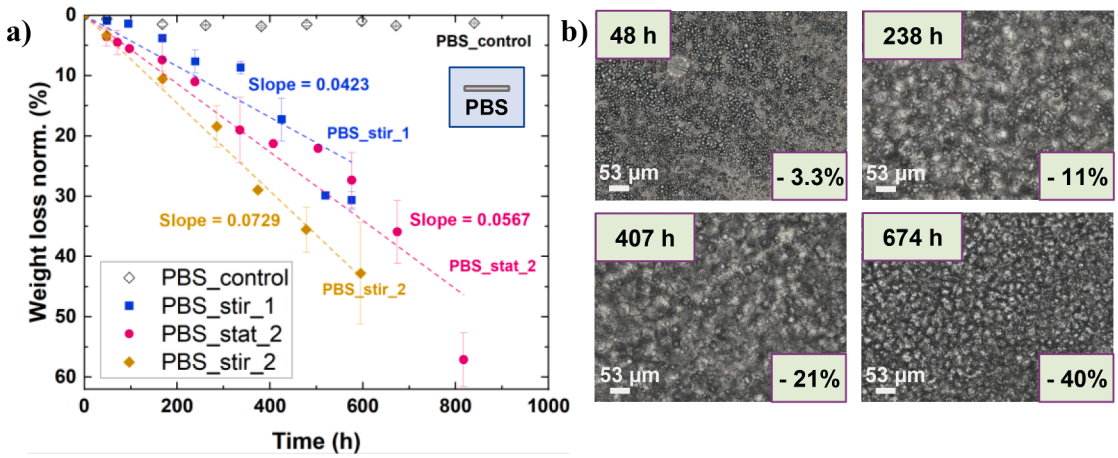


Fig. 1. **a)** Determination of the weight loss for PBS films immersed in aqueous lipase solutions under different experimental conditions. For comparison, slope values are reported within the figure, which are related to degradation kinetics. Dashed lines show the linear fitting of the curves. **b)** Optical profilometry images were taken at 20x magnification for PBS_stat_2 films. In the upper left corner, the degradation time is included (in hours [h]), whereas the weight loss percentage is shown in the lower right corner.

weight of the degraded samples were also monitored as a function of time, and the results are shown in Fig. 2a-b. Regarding PBS crystallinity, DSC analysis revealed no significant changes in the degree of crystallinity (Fig. 2a). For PBS_stir_1 films, there was a small increase in the degree of crystallinity from 28 to 32%, which could be due to a degradation of the amorphous part of the films [50]. Nonetheless, this trend was not observed for films studied under stationary conditions, PBS_stat_2, as the crystallinity remained almost constant. This result implies that the enzymatic degradation occurs simultaneously both in the amorphous and crystalline regions of the film, as a non-detectable change in crystallinity was observed. In the case of PBS_stir_1 films, the degradation could be led by a more preferential attack on amorphous regions, which could explain the small increase in crystallinity. This effect could be due to the agitation of the enzymatic medium: in a static assay, the enzyme finds it easier to reach both amorphous and crystalline regions; when the experiment is carried out with stirring, the enzyme is less prone to attach to the surface, reaching preferentially the more amorphous regions, which are more susceptible to enzymatic attack because of their more porous structure. This equilibrium/slight increase was also observed in the literature on PBS films and fibers [50,51]. In an interesting study with PBS single crystals, AFM showed a preferential enzymatic attack on the crystal edges, whereas the central part remained invariable, giving credibility to a less favored affinity towards crystalline parts [52]. Likewise, the molecular weight of the films, M_n, showed a slight decrease (confirmed by both GPC and ^{1}H-NMR) throughout the course of the enzymatic degradation assay (Fig. 2b), which suggests the bulk PBS structure remained unaltered, indicating that the degradation takes place mainly at the surface, through a surface erosion mechanism, which is in consonance with the crystallinity results provided by DSC.

Traditionally, enzymatic degradation has been considered to follow a surface erosion mechanism, as opposed to a bulk erosion behaviour of hydrolytic degradation. For both mechanisms, crystallinity, molecular weight, and chemical structure, among other factors, are known to influence the degradation. However, in enzymatic degradation, due to its eroding mechanism, the molecular weight of the substrate does not represent an important variation, as the low M_w products are water-soluble [53]. In fact, it has been reported that *P. cepacia* enzymatic degradation of PBS gives rise to 4-hydroxybutyl succinate as the main product (water-soluble), and traces of succinic acid and 1,4-butanediol as well [51,54]. Thus, the results obtained by GPC support that the most probable mechanism for degradation is through surface erosion, as the molecular weight of the degraded films did not change over the experiment. Nonetheless, this type of mechanism usually leads to a decrease in the size of the sample by creating visible holes or eroding the edges [17,44]. In our case, no holes nor reduction in size were observed, but only a decrease in the thickness for highly degraded films. The thicknesses for PBS_stir_1 and PBS_stat_2 films decreased from 120 ± 20 µm (non-degraded films) to 46 ± 6 µm (62% reduction) and 38 ± 5 µm (68% reduction), respectively. Similar observations were reported for

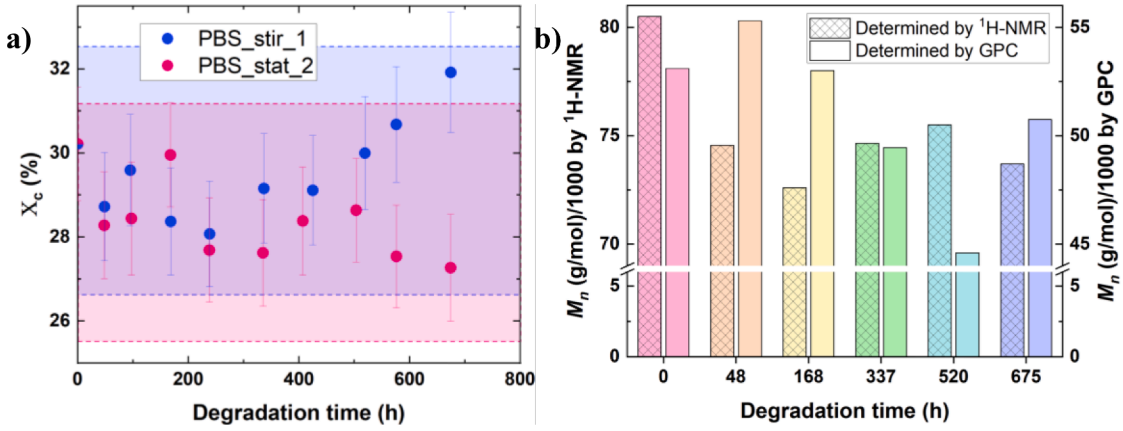


Fig. 2. **a)** Changes in crystallinity of different PBS films, and **b)** Molecular weight (M_n) variation of PBS_stir_1 films (determined both by ^{1}H-NMR, left axis; and GPC, right axis). Shaded areas in Fig. 2a show a ± 10% variation in X_c.

PCL and PLA films [48,55,56].

3.2. Modulation of the enzyme-induced degradation of PBS films through a LbL polysaccharide coating

3.2.1. Fabrication and morphological characterization of Alg/Chi multilayer spray-coated PBS films

The enzyme-induced degradation of PBS films was modulated by coating the hot-pressed neat PBS films with a polysaccharide polyelectrolyte multilayer coating composed by alginate (Alg) and chitosan (Chi), through the spray-assisted LbL technique (Scheme 2a). Three different samples were prepared by varying the number of Alg/Chi layers coating the PBS films, PBS_8L, PBS_24L, and PBS_40L. The thickness was determined from the SEM images of the multilayer spray-coated PBS films by measuring the cross-section of the images (Fig. 3a-c). These observations allowed us to determine the thickness of the Alg/Chi multilayer coating over the PBS films (Fig. 3d). The results show an increase in thickness with the number of Alg/Chi deposited layers, from ~228 nm for PBS_8L to ~1640 nm for PBS_40L (see Table S1 in the Supplementary Information). For the sample with the highest number of deposited Alg/Chi layers, the thickness seems to increase more rapidly. This effect is more clearly appreciated when evaluating the thickness of the monolayers. For PBS_8L and PBS_24L films, each individual layer had a mean value of 28 nm/layer, whereas this parameter rose up to 41 nm/layer for PBS_40L multilayer films, with an increase of almost 50%. This exponential behavior was previously observed in literature for Alg/Chi multilayers [31,33], and explained for many other systems in a recent review [30]. The exponential growth of the multilayer is attributed to the high mobility of the chains within the film, occurring perpendicularly to the film growth direction but also in the plane of the film. This effect produces the diffusion of the polymer in and out of the film architecture, leading to an exponential increase in film growth. The surface wettability properties of the neat and Alg/Chi spray-coated PBS films were studied by water contact angle (WCA) measurements. WCA values dropped from ~70° in the case of neat PBS films to WCAs ~25° for Alg/Chi spray-coated PBS films (see Fig. 3d), which can be attributed to the inherent hydrophilic nature of polysaccharides [57], in contrast to the more hydrophobic character of polyesters [58].

3.2.2. Nanomechanical properties of Alg/Chi multilayer spray-coated PBS films

As alginate/chitosan polyelectrolyte complexes are employed for the enhancement of the mechanical and barrier properties of PBS neat films, the nanomechanical behavior of the polysaccharide coatings was evaluated by nanoindentation. Shallow indent depths of 100 nm were chosen in order to minimize the influence of the PBS substrate. However, it is well known that compliant substrates such as PBS ($E' < 1$ GPa) can influence the indentation response of stiffer coatings even at small indent depths, and lower apparent E' and H values can be found [59]. Hence, in order to evaluate the effect of the substrate on the intrinsic properties of the Alg/Chi coatings, silicon wafers were also used as substrates ($E \approx 150$ GPa).

Fig. 4 shows the E' and H values for the Alg/Chi films spray-coated on PBS and silicon. For the case of compliant PBS substrate, it is clearly seen that E' and H values rise as the number of layers increases, and constant values are approached for the thickest films (60 and 80 layers). Such behavior can be clearly attributed to the influence of PBS that produces substrate-affected E' and H values for the thinnest coatings (< 60 layers). The substrate influence is also apparent in the case of silicon wafers. However, in this case, E' and H values are overestimated for the thinnest coatings because silicon is much stiffer than the deposited film. Most interesting is the observation that the E' and H values of the thickest films are the same within error, irrespective of the type of substrate. Hence, one can assume $E' = 6 \pm 2$ GPa and $H = 260 \pm 60$ MPa as the substrate-

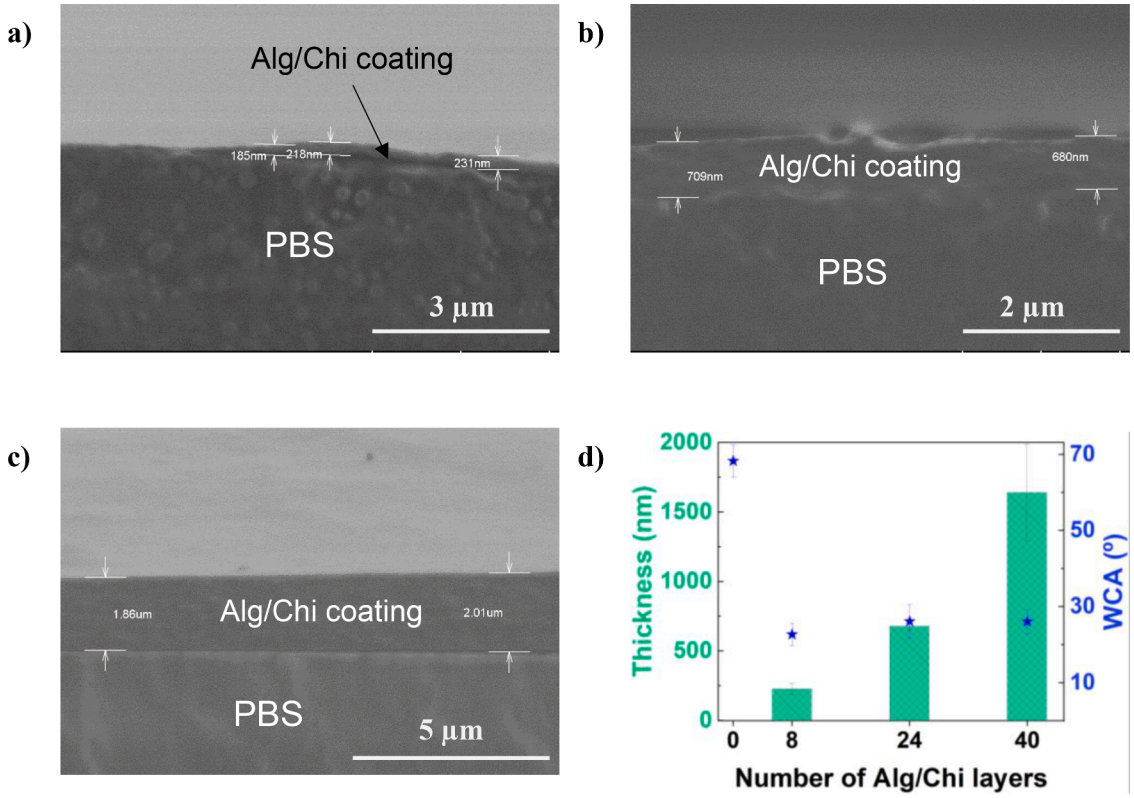


Fig. 3. SEM images of the cross-sections of Alg/Chi coatings spray-coated on PBS films with a different number of layers **a)** PBS_8L, **b)** PBS_24L, **c)** PBS_40L; and **d)** graphical representation of the thickness *vs.* the number of Alg/Chi coating layers (left axis) and water contact angle *vs.* the number of Alg/Chi coating layers (right axis).

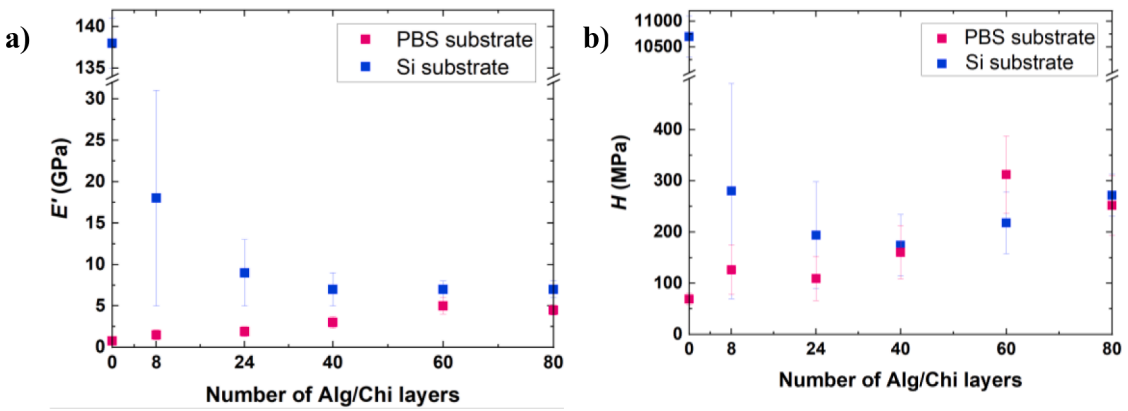


Fig. 4. **a)** Storage modulus (*E'*) and **b)** hardness (*H*) values obtained by nanoindentation for Alg/Chi coatings prepared on PBS films and silicon wafers.

independent values for the Alg/Chi layered system. The fact that such values are in the range of those found for the neat chitosan and alginate films ($E' = 6.0 \pm 0.3$ GPa and $H = 302 \pm 24$ MPa for chitosan; $E' = 8.3 \pm 0.8$ GPa and $H = 306 \pm 52$ MPa for alginate) suggests that the evolution of the deformation field under the indenter takes place with no significant discontinuity across the different layers. In other words, the mechanical behavior of the polysaccharide coating is in agreement with significant interaction between the individual layers. This good interaction between alginate and chitosan layers ensures a durable and stable polysaccharide multilayer coating, which is important to successfully modulate PBS biodegradation.

3.2.3. *Enzymatic degradation of Alg/Chi multilayer spray-coated PBS films*

We now turn our attention to the influence of the polysaccharide Alg/Chi multilayer coatings on PBS film degradation. Fig. 5 shows the results of weight loss degradation curves corresponding to PBS spray-coated samples with a different number of Alg/Chi layers. As can be observed, the degradation seems to present a tendency directly dependent on the number of layers of the Alg/Chi coating. The higher the number of layers, the lower the degradation. This phenomenon is observed both in the weight loss reached and the degradation speed.

Weight loss curves recorded as a function of the time can be fitted to a model to describe the degradation kinetics quantitatively. Generally, for the reactions that involve enzymes, the Michaelis-Menten' model, which describes the kinetics of enzymatic homogeneous reactions that take place in a short period of time (linear section of the reaction), is the most commonly used. For these reasons, as the biodegradation experiments are controlled by heterogeneous reactions for a long time, some corrections were needed in order to describe the whole degradation process, which includes three main steps: adsorption of the enzyme on the PBS surface, degradation of the polymer, and denaturation of the enzyme. In order to accomplish all these requirements, some authors have proposed a model for the enzymatic degradation of PLA that can be described by Eq. (3) [60,61].

$$M_t = \nu_d \cdot \tau \cdot \left(1 - e^{-t/\tau}\right) \quad (3)$$

where m_t refers to the weight loss of the sample (considered similar to W_{loss} indicated in Eq. 1), ν_d stands for the rate of degradation, τ indicates the rate of denaturation of the enzyme (time constant), and implies that the degradation has reached a *plateau* and t is the time (expressed in hours).

The application of the modified Michaelis-Menten' model (Eq. 3) to the experimental data shown in Fig. 5 allows us to extract further information on the effect of the polyelectrolyte coating on the enzymatic degradation of neat PBS films (Table 1).

Regarding the degradation rate of the films, which takes into consideration the first instants of the degradation process, it can be appreciated that this parameter increased as the number of layers of the coating decreased: 0.0848 %/h (PBS_40L) < 0.1003 %/h (PBS_24L) <

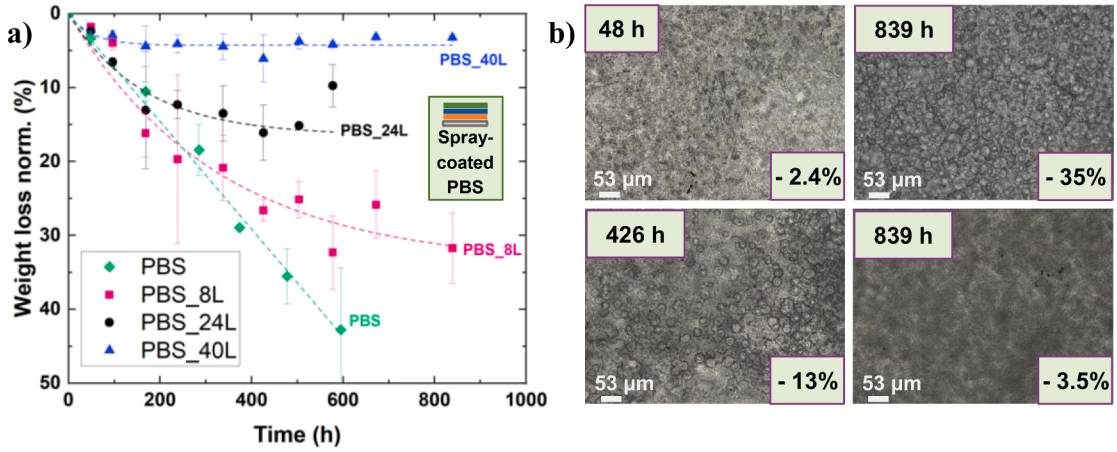


Fig. 5. **a)** Determination of the weight loss for Alg/Chi spray-coated PBS films, PBS_8L, PBS_24L, and PBS_40L, immersed in a lipase aqueous solution (2 mg/mL) under stirring at 37 °C. Neat PBS films are used as a control. Dashed lines show the fitting of the data to the proposed model (Eq. 3). **b)** Optical profilometry images were taken at 20x magnification for the initial steps of degradation: PBS_8L films after 48 h of degradation (top-left image), and at the end of degradation: PBS_8L after 35 days (top-right image), PBS_24L after 18 days (bottom-left), and PBS_40L after 35 days (bottom-right). In the upper left corner of each image, the degradation time is included (in hours [h]), whereas the weight loss percentage is shown in the lower right corner. The surface aspect at the beginning of degradation was similar among all the samples (initial profilometry images of PBS_24L and PBS_8L are shown in Figure S3 in the Supplementary Information).

Table 1
Kinetic parameters of the enzymatic degradation of Alg/Chi spray-coated PBS films. The standard deviation of the mean values is given in brackets.

Sample	ν_d (%/h)	τ (h)	A (%)	R^2
PBS	0.0729 (0.0023)[a]	n.d.[b]	n.d.[b]	0.9953
PBS_8L	0.1045 (0.0164)	325 (84)	34.0	0.9420
PBS_24L	0.1003 (0.0170)	164 (40)	16.5	0.9534
PBS_40L	0.0848 (0.0221)	50 (15)	4.3	0.9375

[a] Obtained from the linear fit of the data related to the kinetics of PBS neat films degradation.

[b] These data could not be obtained for PBS neat films, as the weight loss curves did not reach a *plateau* regime; thus, the lipase is still degrading by the end of the experiment (the rate of denaturation of the enzyme, τ, will be higher than the total time of the experiment: $\tau > 1000$ h).

0.1045 %/h (PBS_8L). These degradation rates are slightly higher than the degradation kinetics obtained for neat PBS films. Nevertheless, concerning the rate of denaturation of the lipase, an inverse relationship with the number of layers can be observed: 50 h (PBS_40L) < 164 h (PBS_24L) < 325 h (PBS_8L). As the neat PBS films did not reach the *plateau* during the weight loss assays (i.e., the lipase continued degrading after concluding the experiment), it was not possible to determine the value for the rate of denaturation of the enzyme (τ) because the model did not apply for this case. For the Alg/Chi spray-coated PBS films, τ was reduced to an inversely proportional extent toward the number of layers of the coating. Finally, the degree of degradation at infinite time (pre-exponential term A), –calculated by multiplying the degradation rate (ν_d) by the rate of denaturation of the enzyme (τ)–, was found to be maximum for PBS_8L samples, with nearly 34% reached, whereas it decreased to values of ~4% for PBS_40L. Overall, the Alg/Chi spray-coated PBS films reached a degradation *plateau* progressively and followed a decreasing order with the number of layers: firstly PBS_40L, then PBS_24L, and finally PBS_8L. It is hypothesized that this sort of modulation of the enzymatic degradation of PBS films by the application of an Alg/Chi multilayer coating could be due to the hindered diffusion of the free enzyme throughout the polysaccharide coating. A similar approach was employed to modulate the drug release from polysaccharide capsules and gels produced through LbL assembly. The incorporation of additional layers hindered drug diffusion, as these added layers constituted a physical barrier to the drug diffusion [42,62].

The stability of the Alg/Chi coating was previously checked thanks to the preparation of Alg/Chi coatings with different layers containing magnetic nanoparticles (Fe_3O_4 NPs). These coatings presented a dark-brownish tone due to these NPs, which remained present (only small cracks were observed on the surface of the films) in the degraded films up to 300 days of enzymatic biodegradation assays (see Figure S4 in the Supplementary Information). Regarding morphological changes on degraded samples, SEM images were taken of the cross-section of the films at the end of the biodegradation experiments (see Figure S5 in the Supplementary Information). In this case, the thicknesses of the degraded PBS films were reduced by the end of the experiments: a small decrease of ~20% in the case of PBS_24L and PBS_40L films and a significant ~70% reduction in the case of PBS_8L films (see Table S2 in the Supplementary Information). These observations were in accordance with the weight loss curves shown in Fig. 5. Furthermore, a partial detachment of the multilayer coating was appreciated by the end of the enzymatic biodegradation assays, related to the experimental conditions. This was evidenced both by the cross-sectional SEM images of the Alg/Chi coatings (see Figure S5) and the optical profilometry images of the Alg/Chi coatings (see Fig. 5) and the Alg/Chi with NPs coatings (see Figure S4).

3.3. Self-degradable PBS films containing enzyme-embedded polysaccharide beads

The observed modulation of PBS enzymatic degradation through the addition of Alg/Chi multilayer coatings was further explored in a different approach by the preparation of self-degradable PBS films by extruding a mixture of PBS pellets containing enzyme-embedded polysaccharide beads (Scheme 2b). The morphology of the lipase-embedded alginate beads employed for PBS self-degradation is shown in Fig. 6a. The particle diameters were found to be ~2 mm with smooth surfaces and some scattered regions that showed a porous morphology.

As a first step, the feasibility of employing the lipase enzyme (in free form) at the temperature conditions required to prepare the PBS films (temperature above the melting point of PBS ~115 °C) was studied. To this aim, the enzymatic activity of the lipase was determined at different concentrations in solutions maintained at room temperature and at 125 °C for 10 minutes. As can be observed in Fig. 6b (left blue-shaded area of the graph), the lipase in solution loses the enzymatic activity when maintained at 125 °C for a short time, which prevents the use of the free enzyme in the preparation of self-degradable films. In contrast, alginate beads with embedded enzyme retain their enzymatic activity even after heating at 125 °C, as shown in Fig. 6b (right yellow-shaded area of the graph). For alginate beads with a lipase concentration of 1.25 and 2 mg/mL, enzymatic activity after heating was retained over 90% with respect to initial values (~100% for 2 mg/mL lipase concentration). Furthermore, it was also appreciated that the activity increased with the concentration of the lipase inside the alginate beads. Similar observations were obtained for Immobead commercial particles, which exhibited good behaviour towards heating, with identical activity values obtained when measured before and after heating (see Figure S6 in Supplementary Information). Therefore, preserving the enzyme by embedding it inside alginate beads has been proven to be necessary to maintain the enzymatic activity at levels high enough to degrade the polyester.

Then, the degradation of PBS films employed as control was compared with that of PBS films containing lipase-embedded alginate beads (PBS_*AlgEmbLi*). Fig. 6c revealed that the maximum weight loss obtained for lipase-embedded PBS films barely achieved a 5%. This could be attributed, on the one hand, to the low enzymatic activity exhibited by the lipase-embedded alginate beads (*AlgEmbLi*) as compared to that of the free enzyme in solution, as seen in Fig. 6b. In addition, it was found that the enzyme concentration (related to the enzymatic activity) of the *AlgEmbLi* particles was 2 orders of magnitude less (~70-100 times less concentrated) than the free enzyme (i.e., in solution) employed for degradation experiments. These results could be related to the prevention of the encapsulated enzyme to water accessibility, due to the protection of the alginate beads. Future experiments will aim to increase the amount of lipase embedded within the alginate beads and their amount within PBS films in order to increase the extent of self-degradation of PBS films.

4. Conclusions

In this study, we propose two complementary strategies to modulate the enzymatic degradation of PBS films triggered by a lipase from *Pseudomonas cepacia*, which involve, on the one hand, the enzyme-prevented degradation of PBS films by coating them with multilayer polysaccharide Alg/Chi films and, on the other hand, the study of self-degradation of PBS films containing lipase-embedded alginate particles. For comparison, as a first step, we report a systematic study of enzymatic degradation of neat PBS films employing a lipase from *P. cepacia* in solution to ascertain the experimental parameters that influence their degradation in solution. The results showed that the increase in enzyme concentration increased the extent and the velocity of degradation of neat PBS films. Furthermore, stirring of the films within the enzymatic solution was found to favor the enzymatic biodegradation of PBS in contrast to stationary assays. Then, PBS films were coated with

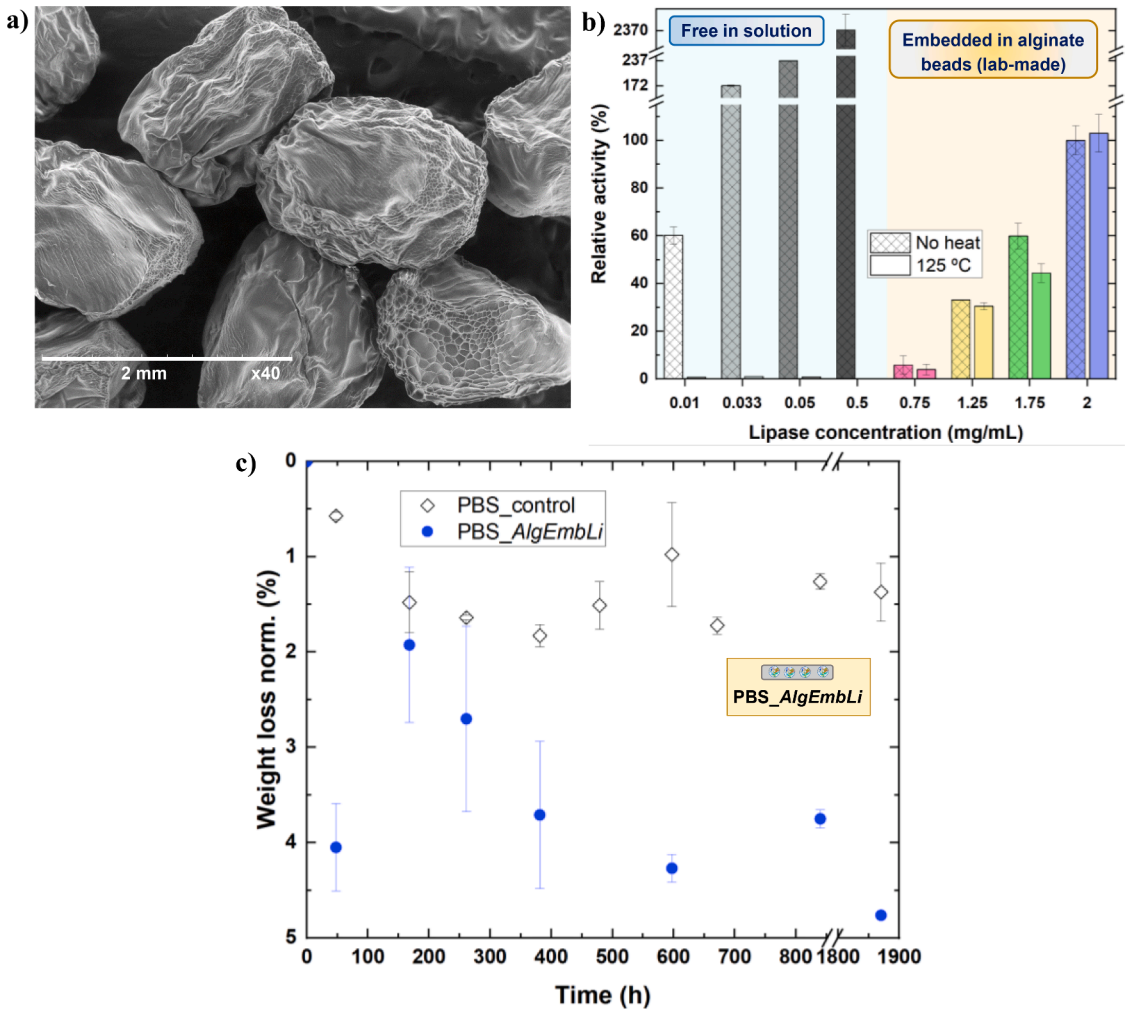
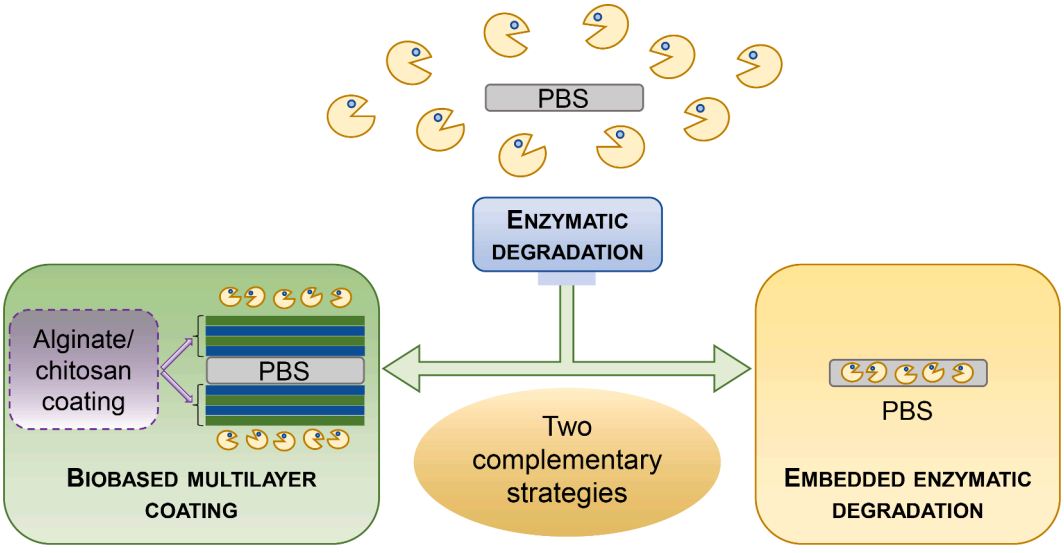
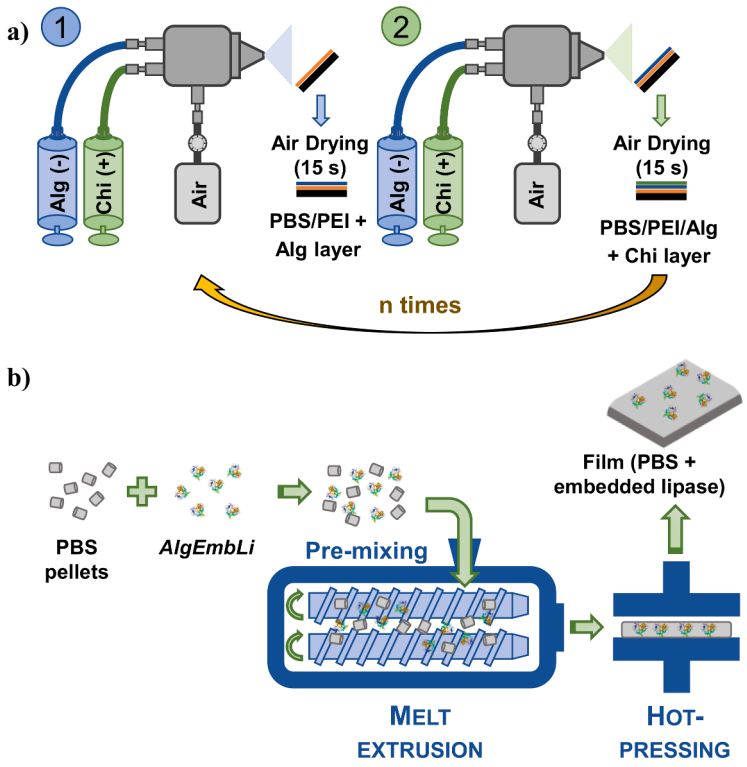


Fig. 6. a) SEM images of the freeze-dried lipase-embedded alginate particles, *AlgEmbLi,* at 40x magnification; **b)** Determination of the relative enzymatic activity (with no-heating treatment and after 10 minutes at 125 °C) of free lipase in solution (blue-shaded area, on the left) and lab-made alginate beads with embedded lipase (*AlgEmbLi*, yellow-shaded area, on the right). The enzymatic activity is normalized to that of the highest lipase concentration (i.e., 2 mg/mL, for embedded-lipase in alginate beads); and **c)** Weight loss degradation curves for self-degradable PBS films.



Scheme 1. Schematic overview of the two complementary strategies proposed in this study for PBS films enzymatic biodegradation.



Scheme 2. Schematic representation of the two strategies used for the preparation of the films: **a)** Spray-assisted layer-by-layer of polysaccharides leading to multilayer spray-coated PBS films, and **b)** Extrusion process of PBS pellets and enzyme-embedded alginate beads (*AlgEmbLi*) to obtain self-degradable PBS films. The spray-assisted LbL process was performed on both sides of the PBS film.

a different number of alginate/chitosan layers by spray-assisted LbL and subjected to enzymatic degradation experiments in solution. The extent of degradation was found to be dependent on the number of coating layers; that is, the higher the number of layers, the lower the degradation rate. The results suggest that the biobased coating constitutes an effective barrier to the diffusion of the lipase, thus proving its effectiveness in modulating the enzymatic activity as a function of the thickness coating. Finally, lipase-embedded alginate beads were employed as enzyme-heating protecting agents for PBS self-degradation. A slight degradation was observed for PBS films containing a certain amount (10 wt% with respect to PBS) of lipase-embedded alginate beads –*AlgEmbLi*–, whereas the control PBS samples showed minor degradation. These results were quite promising, as the enzymatic activity –indicative of the degradation capacity of the enzyme– determined for all these samples was about 2 orders of magnitude lower than that of previous assays.

To sum up, this research expands the knowledge of polyester enzymatic degradation in many ways, studying the most relevant parameters in degradation, its modulation by applying a biobased polysaccharide multilayer coating, and the preparation of self-degradable biobased materials.

CRediT authorship contribution statement

Mario Iván Peñas: Writing – original draft, Writing – review & editing, Data curation, Formal analysis, Investigation, Methodology. **Miryam Criado-Gonzalez:** Data curation, Formal analysis, Methodology, Validation, Visualization. **Antxon Martínez de Ilarduya:** Data curation, Formal analysis, Investigation. **Araceli Flores:** Data curation, Formal analysis, Investigation. **Jean-Marie Raquez:** Supervision, Writing – review & editing, Resources. **Rosica Mincheva:** Methodology, Writing – review & editing. **Alejandro J. Müller:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Rebeca Hernández:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

REBECA HERNANDEZ reports financial support was provided by Ministry of Science Technology and Innovations. ALEJANDRO MULLER reports financial support was provided by Basque Government. ALEJANDRO MULLER reports financial support was provided by European Union.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.polymdegradstab.2023.110341.

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