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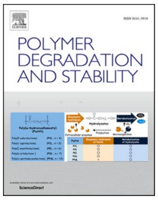

Covalent immobilization of an enzyme on a layered silicate to catalyze the self-degradation of PCL

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ABSTRACT

A lipase from *Burkholderia cepacia* was covalently linked to the surface of Laponite® layered silicate after its activation with glycidoxy moieties on two different routes. The modified silicate was embedded into poly-ε-caprolacton (PCL) for the preparation of self-degradable biopolymers. The activated silicate was characterized by thermogravimetry (TGA) and infrared spectroscopy (FTIR), the location of the linker among the silicate layers was determined by X-ray diffraction (XRD). The activity of the immobilized enzyme was tested in two model reactions, by transesterification in organic medium and hydrolysis in aqueous buffer. The immobilized enzyme was homogenized with the polymer and then films were compression molded at 70 °C. TGA and FTIR measurements verified the successful activation of the silicate but the number of available epoxy groups were limited on the surface. These functional groups linked enzyme molecules to the silicate surface. The enzyme retained its activity even after immobilization and had similar or better catalytic performance than the neat enzyme in both transesterification and hydrolysis. The supported enzyme degraded PCL efficiently, the rate of degradation depended on the type of the linker molecules and on the activated enzyme content of the polymer. The covalently linked enzyme catalyzes the degradation of a solid polymer matrix thus allowing the preparation of self-degradable composites with controlled lifetime and helping the reduction of environmental pollution.

1. Introduction

Continuously increasing attention focuses on the enzymatic degradation of polymers. These biocatalysts increase the degradation rate of biopolymers, mainly aliphatic polyesters, under mild conditions (30–50 °C and close to neutral pH) [1] leading to the formation of non-toxic small molecular weight compounds [2]. Biodegradation prevents the pollution of the oceans by plastics or the contamination of the ecosystem by microplastics. The first studies in this area described the enzymatic degradation of polymers in the solution of the biocatalyst; the polymer samples were immersed into a buffer containing the enzyme. Several polymer/enzyme pairs have been identified for the hydrolysis of polyesters. Proteinase K from *Tritirachium album* accelerates the degradation of polylactic acid (PLA) [2], pseudomonas lipase catalyses the hydrolysis of poly-ε-caprolactone (PCL) [3], while poly(3-hydroxybutyrate) depolymerase facilitates the fragmentation of poly-3-hydroxybutyrate (PHB) [4]. These experiments revealed the role of several parameters influencing the reaction rate of enzymatic degradation including pH [5], temperature [6], ionic strength [5] or the structure of the polymer [7]. Although polymers disintegrate and dissolve in the enzyme solution in some hours or days, but the enzyme was added externally to the polymer in these cases [8], and the approach cannot always be applied like in composting or in the case of resorbable scaffolds.

A more direct approach is the preparation of self-degradable plastics by embedding enzymes into the polymer matrix. Although, polyesters hydrolyze in aqueous medium without any catalyst, and one may call the hydrolysis of these polymers self-degradation, but this process is slow [9] or the pH must be extremely alkaline or acidic for achieving an acceptable rate [10]. Real self-degradable plastics can be prepared by dispersing an enzyme in the polymer matrix. As polymer processing is

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executed at high temperatures or in organic solvents, the enzymes should be immobilized on a carrier. Immobilization stabilizes the active conformation of the enzyme, improves its thermal stability, selectivity or activity [11,12]. Enzymes may be linked to the surface of the carrier by secondary interactions, covalently or they can be entrapped in cross-linked polymers or polymer nanoparticles [13]. A physically immobilized enzyme degrades a polymer in suspension [14] or in polymer fibers or mats prepared by electro-spinning [15]. A self-degradable plastic described earlier contained a neat enzyme [16,17], while in others the enzyme was adsorbed physically on the surface of a nanocarrier [18] or it was entrapped in polymer microparticles [19,20]. Both methods proved efficient in the catalysis of the degradation of polymers and almost complete degradation was achieved in most cases. Compared to this method, covalently linked enzymes cannot desorb from the surface of the carrier and chemical immobilization allows the recycling of the enzyme after degradation. On the other hand, covalent linkage may hinder the formation of the polymer/enzyme complex due to the reduced mobility of the enzyme. In addition, the investigation of the degradation kinetics of self-degradable composites has been neglected until now. Peñas et al. [17] are one of the few authors who studied it but they approached degradation kinetics from the aspect of the degrading polymer (different polymers and blends with the same enzyme) while we try to regulate degradation by the application of a carrier and by changing the immobilization approach.

To the best of our knowledge, the application of an enzyme covalently linked to a solid carrier and embedded in a polymer has not been investigated as a way to produce self-degradable biopolymers yet, thus we explored this approach. Layered silicates have been proven proper carriers for enzyme immobilization in general [21-23] and in self-degradable polymers [18], too. They can be modified by a variety of functional groups by reacting their edge silanol groups with alkoxysilane compounds [24-26]. A possible route for the chemical immobilization of enzymes on the surface of layered silicates is their activation with epoxy groups [27,28]. The epoxy groups can react with the amine, thiol and hydroxyl groups of different amino acid moieties such as lysine, cysteine and serine, respectively, located in enzyme molecules. The reaction directly leads to a stable covalent bond stabilizing the enzyme, fixing its conformation and modifying its activity, thus an epoxy-activated surface could ensure stable one step binding compared to the popular aldehyde-grafted carriers [29]. The benefits of covalently immobilized enzymes have been proven in homogenous reactions [30] but the efficiency of the approach may be questioned if the substrate is insoluble in the reaction medium, as in the case of a water insoluble polymer in water.

The aim of our study was to check the feasibility of the approach of using covalently immobilized enzyme to catalyze the degradation of an aliphatic polyester. Amano lipase PS from *Burkholderia cepacia* (PS) was attached chemically to the surface of Laponite® layered silicate on two different routes and at five different reaction temperatures. The modified silicate and the one containing the immobilized enzyme were characterized by several methods in detail. The effect of the immobilization route and the reaction temperature on the activity of the enzyme was checked by two different model reactions, while its efficiency in catalyzing degradation was also investigated by embedding it into PCL.

2. Experimental

2.1. Materials

The poly-ε-caprolactone used in the experiments was the Capa 6800 grade produced by Perstorp, Sweden. According to the producer, its molecular mass is 80 kDa, melt flow rate (MFR) 3 g/10 min (160 °C, 2.16 kg), while its melting temperature is 58−60 °C. The Amano Lipase PS, from *Burkholderia cepacia* (PS; E.C. 3.1.1.3.; 30,000 U/g, pH 7.0; 50 °C) was received from Sigma Aldrich. The silicate used as carrier was the Laponite® XLG grade (XLG) obtained from Byk Additives and Instruments. The cation exchange capacity of the silicate is 0.55 meq/g. The various reactants and solvents, i.e. (3-glycidoxypropyl)trimethoxysilane (GPTMS) (97 %, Thermoscentific), 3-aminopropyltriethoxysilane (AMPTES) (99 %, Thermoscientific), Ipox RD 18 [1,6-hexanediol diglycidyl ether, (HDGE) Ipox Chemicals Ltd.], toluene (99.97 %, Molar Chemicals), propan-1-ol (a.r., Molar Chemicals), hexane (96 %, Molar Chemicals), propan-2-ol (Reag. Ph. Eur., VWR Chemicals), sodium-dihydrogen-phosphate (puriss, Molar Chemicals), disodium hydrogen phosphate dihydrate (>99 %, Fluka), bovine serum albumin (Sigma Aldrich, >96 %), Bradford reagent (ready to use, Alfa Aesar), methyl-tert-butyl ether (MTBE, Alfa Aesar), racemic-1-phenylethanol (Sigma Aldrich), vinyl acetate (Sigma Aldrich), ethanol (Alfa Aesar), racemic-1-phenylethyl acetate (Sigma Aldrich) were used as received without any further purification. If not otherwise stated, all measurements were carried out at ambient temperature.

To prepare the buffer solution, 30 mmol di-sodium hydrogen phosphate dihydrate and 20 mmol sodium-dihydrogen-phosphate were dissolved in 900 mL distilled water. The exact pH of the buffer was adjusted to 7.0 (for the degradation studies) or 7.5 (for the model reaction) by the addition of 1 M NaOH solution. The pH was checked with a Hanna Edge HI2002 pH meter. The solution was placed into a measuring flask of 1 L volume; it was filled up to sign and homogenized.

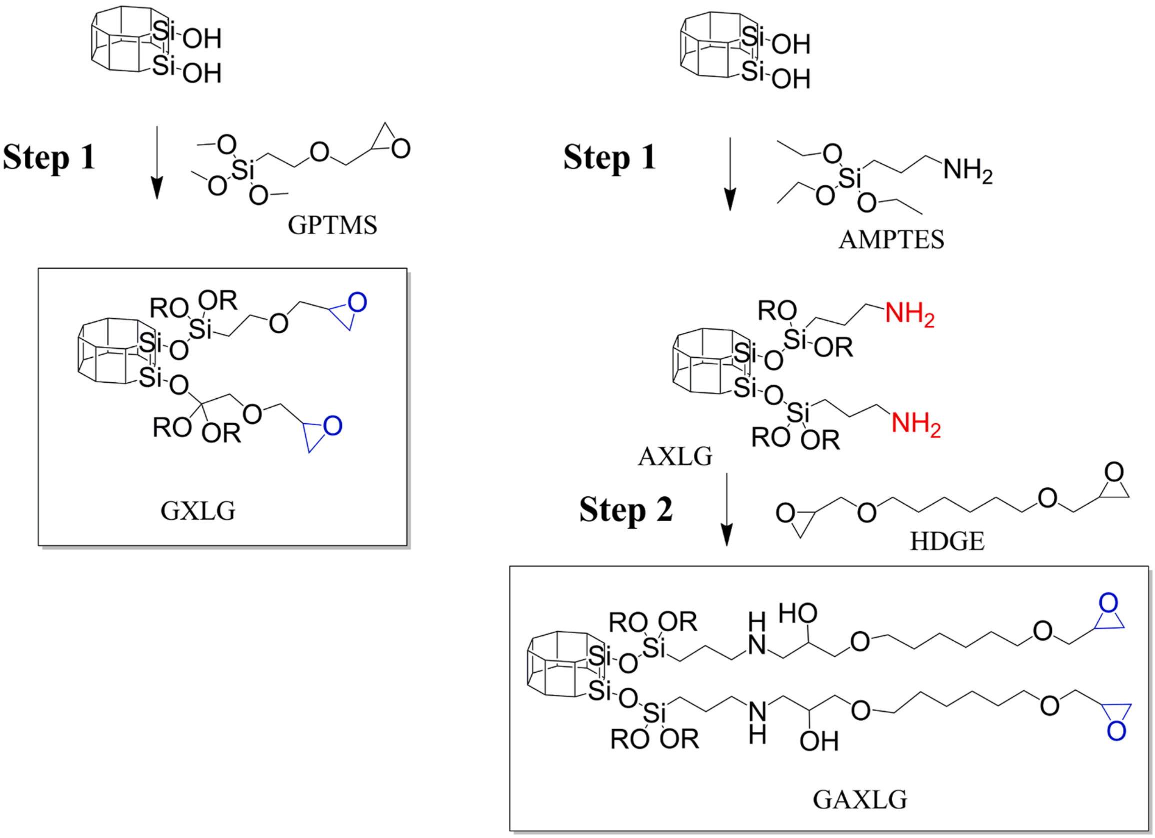
2.2. Sample preparation

Two different routes (Scheme 1) were applied for the activation of the carrier by the epoxide group, which can react with the enzyme. Firstly, different amounts of the silane compound (see Table 1) were dissolved in toluene then the silane solutions were poured onto Laponite® XLG (**Step 1**). The suspensions were stirred by a magnetic stirrer for 48 h, and then they were filtered using a P04 glass filter and washed three times with toluene. The modified silicate was dried in a vacuum oven at 70 °C and 100 mbar. The samples were let to cool in vacuum then they were stored at room temperature. In the next step (**Step 2**), 100 g/L amine functionalized silicate (AXLG) suspension was prepared in 1-propanol and it was mixed with the 1-propanol solution of HDGE (57.5 g/L) at 1:2 ratio resulting in 33.3 g/L AXLG and 38.3 g/L HDGE final concentration. The suspension was stirred for 24 h, it was filtered using a P04 glass filter, and then washed three times with the mixture of 2-propanol/ethanol (1:4). Subsequently, the filtrate was dried at room temperature overnight. GPTMS contains epoxide groups, so the latter reaction did not have to be performed on the GXLG samples. The last step (**Step 3**) was the chemical immobilization of the enzyme in PBS buffer (pH = 7.0) at different temperatures (25, 30, 40, 50, 60 °C) with continuous stirring at 250 rpm for 24 h. 2 g GLXG or GAXLG samples were immersed into 100 mL solution containing lipase PS (10 g/L). After the reaction, the solid was let to settle. The sediment was filtered using a P04 glass filter, washed three times with 2-propanol and then three times with hexane. Finally, the powder samples were dried at room temperature in 24 h then they were stored at 4 °C. The modified silicates are identified as *l*PS-GXLG and *l*PS-GAXLG, according to the linker moiety.

In order to prepare the polymer containing the Laponite® linked with the enzyme, PCL was cooled to liquid nitrogen temperature and then ground to powder. The ground PCL was sieved and particles smaller than 500 μm were homogenized with the enzyme modified silicate by vigorous shaking in a tube. The powder mixture was compression molded into films of 200 μm thickness at 70 °C and 130 bar pressure in 2 min using a Fontijne SRA 100 apparatus. The films were cooled in ice water and then stored at 4 °C. PCL composites with different silicate contents (2.5, 5, 10 vol%) were prepared both from *l*PS-GXLG and *l*PS-GAXLG produced at the immobilization temperature of 25 °C.

2.3. Characterization, measurements

The silicates modified with the silane compounds were characterized



Scheme 1. Reaction schemes of the activation of Laponite® with epoxide functionality in a one-step method with GPTMS (link) and in a two-step method with AMPTES and HDGE (right). Carriers for ready-to-use are framed.

Table 1
Composition of the reaction mixture during the three steps of enzyme immobilization.

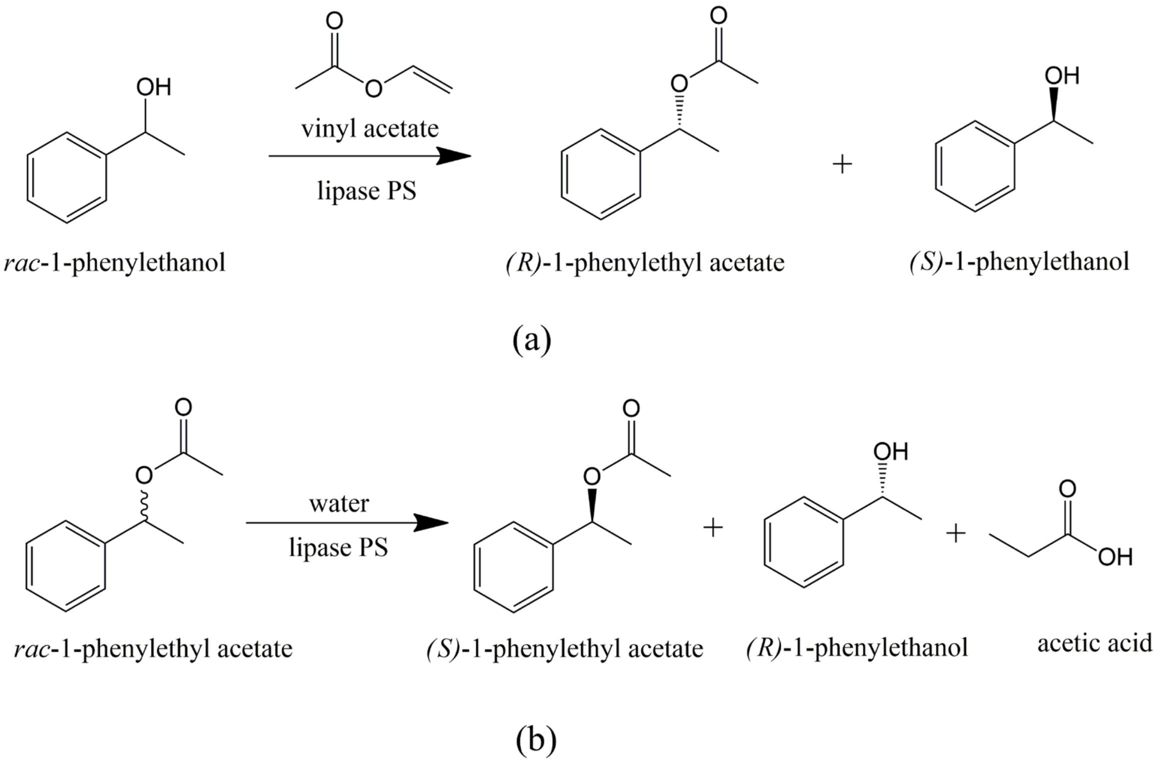
Sample	Reagent	Step 1		Step 2		Step 3	
		XLG (g/l)	Silane (mmol/l)	Silicate (g/l)	HDGE (g/l)	Silicate (g/l)	Lipase (g/l)
*l*PS-GXLG	GPTMS	120	480	–	–	20	10
*l*PS-GAXLG	AMPTES	120	480	33.3	38.3	20	10

GXLG: Laponite® XLG coupled with (3-glycidoxypropyl) trimethoxysilane; GAXLG: Laponite® XLG coupled with 3-aminopropyltriethoxysilane and reacted with 1,6-hexanediol diglycidyl ether; *l*PS-GXLG: lipase PS linked to GXLG; *l*PS-GAXLG: lipase PS linked to GAXLG.

by thermogravimetric analysis (TGA) (Perkin Elmer TGA6) in oxygen. The samples were heated from 50 to 800 °C at 10 °C/min, and then they were held at 800 °C for 10 min. The gallery structure of the silicate was studied by X-ray diffraction using a Philips X-pert diffractometer in the range of 2–12° 2θ angles with CuK$_\alpha$ radiation (0.154 nm) at 40 kV and 35 mA anode excitation. The attachment of the silanes to the silicate was analyzed by Fourier-transform infrared spectroscopy (FTIR) in KBr pastilles containing 1 mg sample in 250 mg KBr. The spectra were recorded using a Bruker Tensor 27A apparatus in the 4000 and 400 cm^{-1} wavenumber range at 2 cm^{-1} resolution and 64 scans.

The lipase content of the enzyme preparation was quantified with the Bradford reagent. 500 µl enzyme solution was pipetted into 2250 µl Bradford reagent. The UV-Vis spectra of the mixture was recorded using a Unicam UV-500 apparatus between 350 and 700 nm at 600 nm/min scan rate and 1 nm resolution in a glass cuvette of 1 cm thickness. The activity of the immobilized enzyme was checked by two different model reactions, transesterification and hydrolysis (Scheme 2). In the first model reaction the transesterification of racemic-1-phenylethanol (50 µl) with vinyl acetate (100 µl) in hexane/MTBE mixture (2/1 volume ratio, 1 mL) was catalyzed by the immobilized or the neat enzyme (10 mg). The reaction mixture was shaken at 30 °C for 18 h. After the allotted time, 50 µl of the solution was diluted with 1000 µl ethanol, and then it was injected into an Agilent 4890D gas chromatograph. The apparatus was equipped with a FID detector and a Hydrodex β-6TBDM column [25 m × 0.25 mm × 0.25 µm film with heptakis-(2,3-di-O-methyl-6-O-t-butyldimethylsilyl)-β-cyclodextrine; Macherey & Nagel] using H_2 as carrier gas (injector: 250 °C, detector: 250 °C, head pressure: 12 psi, split ratio: 50:1). In the second model reaction, 10.0 mg biocatalysts (Lipase PS immobilized on Laponite® XLG carrier or native Lipase PS) was measured into a 4.0 mL glass vial to which 1.0 mL reaction medium was added (50 µL racemic-1-phenylethyl acetate dissolved in the mixture of 800 µL sodium phosphate buffer 50 mM, pH 7.5 and 200 µL 2-propanol). The mixture was shaken at 37.0 °C by an orbital shaker (Heidolph Titramax 100, at 850 rpm) for various times. Samples (20 µL) after 2, 4 and 18 h were taken, and then the composition of the reaction medium was analyzed by gas chromatography with the method described above.

Thermal properties of the composites were characterized by thermogravimetry and differential scanning calorimetry. The detailed experimental data and results are presented in the Supplementary Information.



Scheme 2. Model reactions used for the determination of enzymatic activity; a) transesterification in a MTBE/hexane mixture, b) hydrolysis in aqueous medium.

2.4. Degradation studies

The films were cut to pieces of the approximate weight of 40 mg, their exact weight was measured and then they were placed into glass vials containing 10 mL phosphate buffer. Degradation was carried out at 40 °C on three parallel specimens. The samples were taken from the solution at various intervals, washed, wiped dry, and then weighed. The amount of products dissolved into the degradation medium was determined by UV–Vis spectroscopy using the same apparatus as before, the spectrum of the mixture was recorded between 200 and 600 nm.

3. Results and discussion

The results are presented in several sections. The activation of the silicate and the immobilization of the enzyme are presented first, and then the results of the degradation experiments are discussed in the next section. The kinetics of degradation and its modeling are described in the final section of the paper.

3.1. Activation of the silicate

Different methods were used to prove the successful modification of the silicate and the linking of the enzyme to it. First, TGA measurements were carried out to estimate the conversion of the silylation reaction and the reaction of the amino-modified silicate (AXLG) with HDGE. Weight loss measured in the temperature range between 150 and 800 °C results from the release of the water from the interlamellar space and the degradation of the organic compounds bonded to the surface [31]. TGA traces recorded on the neat Laponite®, the Laponite® modified with 400 mmol/100 g GPTMS (GXLG) or AMPTES (AXLG), and on the latter after the reaction with HDGE (GAXLG) are presented in Fig. 1. The results obtained on the coated samples were corrected with the weight loss measured on the neat, non-reacted silicate, which loses adsorbed water (see Eq. 1).

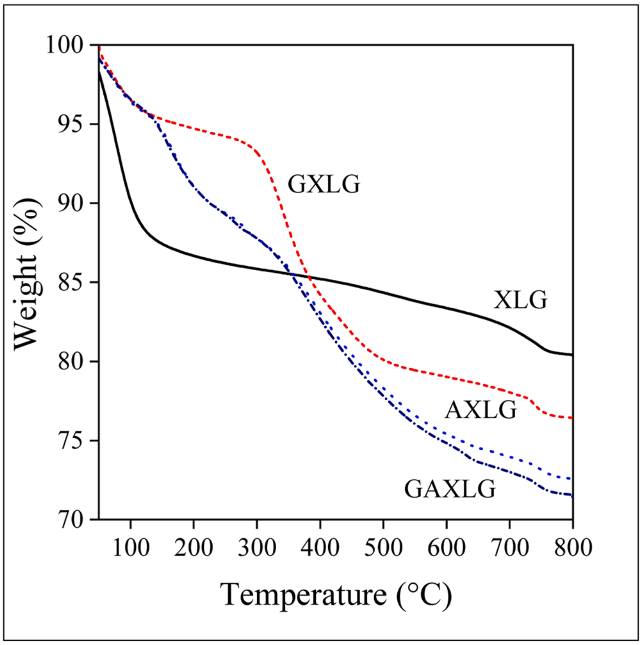


Fig. 1. TGA traces recorded on neat Laponite®, silylated with GPTMS (—), with AMPTES (···) and after the reaction of this latter with HDGE (-----).

$$c_{org} = wl_{mod,\ 250-650\ ^\circ C} - \frac{wl_{Lap,\ 250-650\ ^\circ C} \cdot w_{mod,\ 650\ ^\circ C}}{w_{Lap,\ 650\ ^\circ C}} \qquad (1)$$

where c_{org} (%) is the the organinc content of the modified silicate, $wl_{mod,\ 250\text{–}650^\circ C}$ (%) and $w_{Lap,\ 250\text{–}650^\circ C}$ (%) are the weight loss of the modified and the neat silicate respectively between 250 and 650 °C, while $w_{mod,\ 650^\circ C}$ and $w_{Lap,\ 650^\circ C}$ (%) are the remaining mass of the modified and neat silicate respectively at 650 °C.

The organic content of the samples was 12.6 and 11.9 wt% after the silylation reaction while 12.9 wt% for the GAXLG sample (reaction product of AXLG and HDGE). If we assume that all three alkoxy groups of the silane compounds hydrolyze, and the molecular weight of the organic compounds bonded to the surface is 113 and 58 g/mol for the 3-glycidoxypropyl and the aminopropyl moieties on GPTMS and AMPTES, respectively, 111.4 mmol GPTMS and 205.8 mmol AMPTES are on the surface of 100 g silylated silicate. We must emphasize here that 111.4 and 205.8 mmol are the largest possible amount of epoxy and amine groups, respectively, attached to the surface as we assumed that all the ethoxy or methoxy groups hydrolyze. This number equals the maximum number of epoxy and primary amine groups attached to the surface. However, not all of these functional groups react with the enzyme in the case of GXLG and with HDGE in the case of AXLG, since silane moieties may be located not only on the outer surface of the particles or silicate disks but in the interlamellar space, too. TGA measurements show that only a small fraction of the attached amine groups is accessible for the HDGE molecules in AXLG. The organic content of AXLG increased from 11.9 % to 12.9 % after the reaction with HDGE (GAXLG), i.e. 100 g GAXLG contains 0.99 g HDGE. As the molecular weight of HDGE is 230.3 g/mol, about 4.3 mmol amine groups could react with HDGE (Scheme 1, Step 2) resulting in 4.3 mmol surface epoxy groups accessible for the enzyme in **Step 3** on 100 g silicate. (The initial molar ratio of HDGE was 500 mmol to 100 g silicate in the reaction medium (Table 1) that indicates that ~0.86 % of HDGE reacted with the silicate).

According to the XRD measurements, a large part of the silane molecules containing inaccessible amine groups is located in the interlamellar space. Fig. 2 shows the X-ray diffractograms of Laponite® XLG, its silylated forms (GXLG, AXLG) and GAXLG. Gallery distance increased after modification from 1.32 nm to 1.6 nm and to 1.94 nm in the case of GXLG and AXLG, respectively. Increasing layer distance might result from the diffusion of toluene or the silane reagents into the galleries. The thickness of XLG disks is 0.92 nm while their distance depends on the moieties (sodium ions, interlamellar water) between them. Layer distance did not increase because of toluene diffusion as the XRD traces recorded on a sample immersed into toluene without the silanes (not shown) and that of the neat Laponite® are the same. (The TGA traces of these two samples were also identical.) This result indicates that water is not exchanged by toluene in the interlamellar space; water cannot diffuse from the interlamellar space into toluene. Consequently, the increase in layer distance is due to the interlemellar adsorption of silane compounds. The layer distance of GXLG suggests a monolayer arrangement of the silane compound among the disks. The increase in interlamellar distance caused by AXLG is twice as much as in the case of GXLG indicating a double layer arrangement. The small amount of reacted HDGE shows that just a small number of amine groups could react with HDGE, the most of the silane compound is located in the interlamellar space, and thus it is not available for HDGE. The number of accessible epoxy groups of GXLG is difficult to estimate. The monolayer arrangement among the discs indicates a larger amount of organic molecules on the outer surface of the silicate than in the case of AXLG thus also a larger number of accessible epoxy groups for the enzyme. However, even this amount might be much below the 111.4 mmol/100 g determined by TGA.

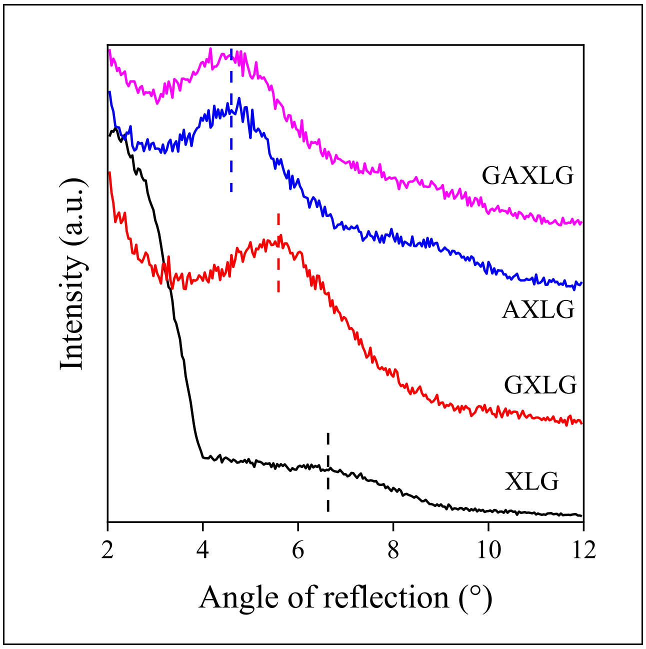


Fig. 2. XRD patterns of neat Laponite®, GXLG, AXLG and GAXLG.

The successful attachment of the silane was also verified by FTIR spectroscopy; the spectra of the modified silicates are shown in Fig. 3. The spectra are dominated by the most intensive peaks of the silicate (Si–O– vibration at 1006 cm^{-1}, Si–O–Mg and Si–O–Si at 466 and 446 cm^{-1}, respectively, and Mg_3OH at 653 cm^{-1}), but the characteristic peaks of the silane molecules also appear in the spectra [32]. The stretching vibration of methyl and methylene groups around 2900 cm^{-1} are visible in the spectra of GXLG, AXLG and GAXLG. The Si–O–C vibration of the methoxy or ethoxy group should appear at about 1070 cm^{-1}, but neither a peak nor a shoulder can be seen close to the peak at 1006 cm^{-1}. The lack of the shoulder indicates the absence of ethoxy or methoxy groups on the silicate surface after its reaction with the silanes that justifies our hypothesis presented during the evaluation of the TGA results claiming that all methoxy and ethoxy groups hydrolyze during their reaction with the silicate.

The characteristic peaks of the amine group appear at around 3400, 1571, 1495, 1337 [33] and 785 cm^{-1} [34] in AXLG and GAXLG. The spectra of the latter two samples cannot be distinguished because of two reasons. The peak of primary and secondary amines overlap with each other. Although the occurrence of the reaction could be proven by a shift of the amine peaks, the number of reacted amine groups is small compared to the remaining primary amines, which makes the detection of any possible shift impossible.

Ethers usually have simple spectra. The only characteristic peak of alkaline ethers at about 1100 cm^{-1} is assigned to the antisymmetric C–O–C stretching vibration [34] but it is hidden by the peak of the SiO– bond at 1006 cm^{-1}. The peak of cyclic ethers, i.e. epoxy ring has their most characteristic peak at 860 cm^{-1} [34], which is superimposed on the SiO– peak (Fig. 3).

The results presented in this section confirm that glycidyl-ether groups are located on the surface of the silicate allowing the chemical linking of the enzyme to it. Unfortunately, the recorded FTIR spectra cannot be analyzed quantitatively. The chemical structure of the two silanes and HDGE differ from each other, the neighboring amine, epoxy and ether groups influence the absorption coefficients and the exact location of methyl and methylene peaks thus the amount of the available glycidyl-ether groups cannot be estimated with acceptable accuracy.

3.2. Immobilization of the lipase

Epoxy groups of the activated silicate can react with the lysine (Lys), cysteine (Cys) and serine (Ser) moieties of the enzyme (Fig. 4.). Modified silicates were characterized after enzyme immobilization again with TGA, XRD and FTIR, but these methods cannot detect the presence of the enzyme on the surface of the silicate because of its small concentration. This is not very surprising if we consider the fact that enzyme preparations purchased from chemical producers usually contain about 5 % enzyme and a large quantity of other materials used for the stabilization of the preparation. The real enzyme content of the purchased preparation is smaller than 2 wt% determined by the Bradford method [35]. Small changes could be observed in the structure of the silicate by XRD

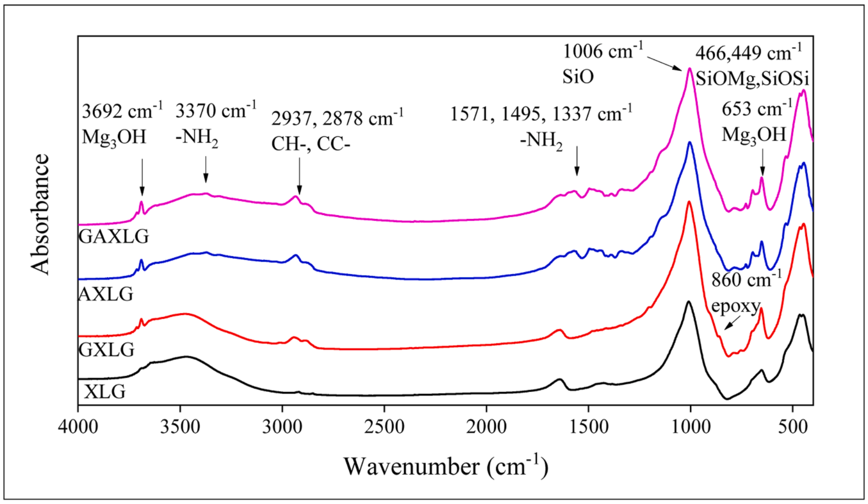


Fig. 3. FTIR spectra of neat Laponite®, GXLG, AXLG and GAXLG with the characteristic peaks.

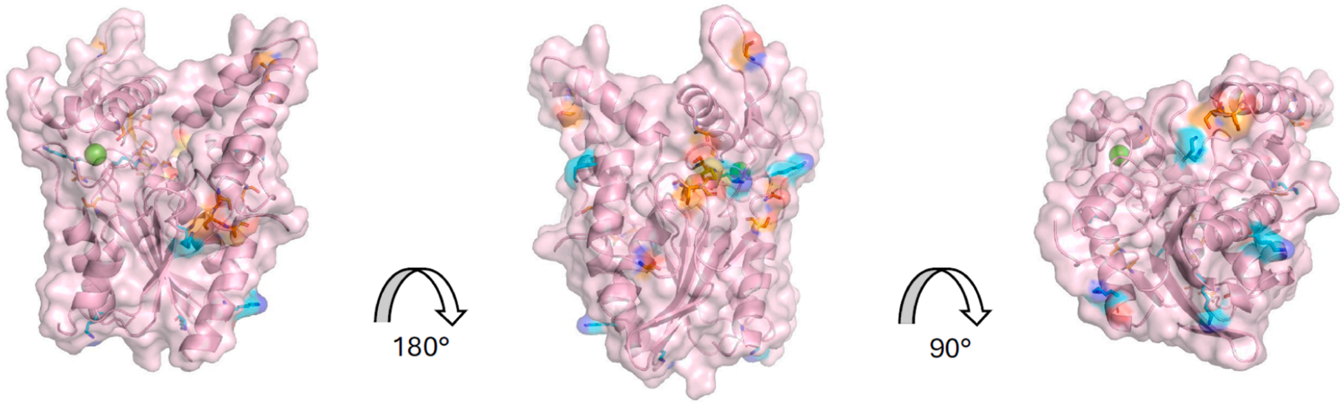


Fig. 4. Structure of lipase PS (PDB ID: 2LIP). Cys (yellow), Ser (orange) and Lys (cyan) moieties located on the surface of the enzyme are displayed in the figure (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

(not shown here) for *l*PS-GXLG and *l*PS-GAXLG, the regularity of the layers decreased but this might be caused by the aqueous medium of the enzyme immobilization step.

The immobilization of the enzyme was proven by the measurement of its activity on the silicate samples in the back and forth reaction pathways using the corresponding racemic secondary alcohol and acetate to cover the industrially relevant reaction routes of lipases. The reactions presented in Scheme 2 were followed by gas chromatography to compare the biocatalytic activity (U_B, U/g) of the samples prepared at different temperatures. Representative chromatograms are collected in the Supplementary Information. The results of the measurements show the amount of the product [(R)-1-phenyletylacetate; μmol or 1-phenylethanol; μmol] formed by 1 g biocatalyst in 1 min. The optimum conditions of lipase PS is pH 7.0 and 50 °C as given by the producer. Consequently, one would expect that enzymatic activity increases with increasing immobilization temperature and a maximum is observed in activity at 50 °C because enzyme conformation is optimal under that condition. The enzyme does not dissolve in the reaction medium (mixture of hexane and MTBE) of the transesterification reaction, thus the measured activity values characterize the immobilized enzyme. This reaction mode is often applied in industry, so the results have relevance in synthetic biocatalysis, as well [36]. Contrary to our expectation, the enzyme loses activity with increasing immobilization temperature and maximum activity was obtained for the samples prepared at room temperature (Fig. 5). As the neat enzyme cannot dissolve in the reaction mixture, it forms aggregates and the substrate molecules can react only with a limited number of enzyme molecules. The Laponite® carrier helps the dispersion of the enzyme in the reaction mixture leading to larger conversion and enzyme activity. Conversion was 13.1 % with the neat enzyme in transesterification, while its immobilized form, i.e. GXLG and GAXLG, resulted in 13.5 % and 27.3 %, respectively, at room temperature. We must emphasize here that 10 mg neat enzyme and 10 mg enzyme containing silicate was compared in the test. However, the amount of the enzyme added was half of the weight of the silicate meaning smaller amount of immobilized enzyme shows similar or larger activity than the neat preparation. Decreasing enzymatic activity with increasing immobilization temperature can be explained by the fact that the producer did not offer any information about the length of time tolerated by the enzyme at the optimum temperature. Longer times might result in the denaturation of the enzyme at higher temperature, especially if some region of the enzyme is already linked to the silicate while other sections move faster due to the elevated temperature thus increasing the stress on the enzyme. Based on the results, the conclusion can be drawn that maximum enzymatic activity is achieved by immobilization at ambient temperature and it equals (*l*PS-GXLG) or exceeds (*l*PS-GAXLG) the activity of the neat enzyme in the transesterification reaction.

The hydrolytic degradation of the polymers takes place in aqueous medium, so the immobilized enzyme was characterized also by hydrolysis. The aqueous medium of the hydrolysis dissolves the enzyme; both

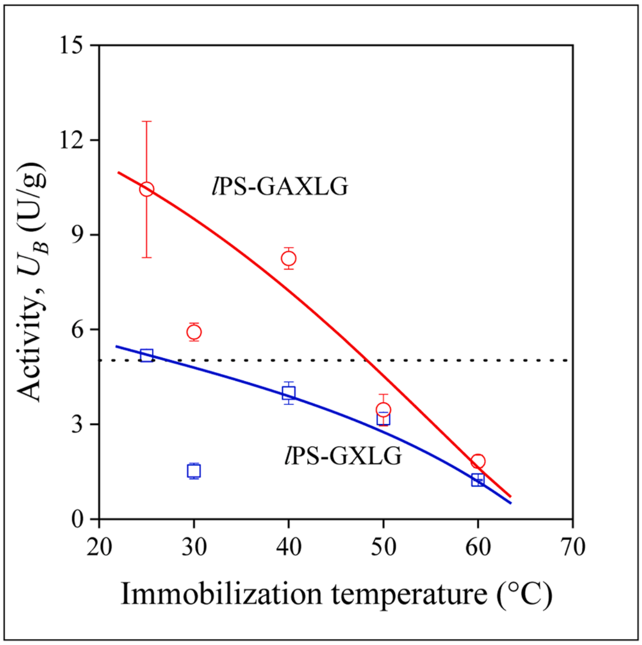


Fig. 5. Enzymatic activity of the lipase (U_B) immobilized on GXLG (□) and GAXLG (◯) at different immobilization temperatures in the transesterification model reaction (Scheme 2a). The dashed horizontal line is the activity of the neat enzyme.

the enzyme and the substrate are dissolved promoting the collision of the reactants. Fig. 6 presents the conversion of the hydrolysis reaction as a function of time for the neat enzyme, *l*PS-GXLG and *l*PS-GAXLG prepared at room temperature. The neat enzyme dissolves practically immediately and catalyzes the hydrolysis of 1-phenylethyl acetate, which is completed in minutes. The reaction is slower in the case of the immobilized samples, since the reaction is heterogeneous because the enzyme does not dissolve in the reaction medium. The final conversion (35.0 % and 23.1 % in the case of GXLG and GAXLG, respectively, immobilized at room temperature) and the enzymatic activity of the immobilized enzyme (Fig. 7) are inferior to those of the neat enzyme (43.3 %) but the probability of the substrate finding the active site of the enzyme is smaller in a heterogeneous than in a homogeneous reaction. As the rate of the reaction is proportional to the concentration of the dissolved reactant, this decrease results from a decrease in phenylethyl acetate concentration as a function of conversion. The attention must be called here to the fact that the two different activation methods used result in different tendencies. Larger enzymatic activities have been measured for GAXLG than GXLG in the transesterification reaction, while GXLG performed better in the catalysis of hydrolysis. The rate of enzyme reaction depends on the number of the available active centers of the enzyme, which is determined by the dispersion of the carrier in the reaction mixture and the number of enzyme molecules on the carrier surface. The linker in GAXLG is longer and contains a flexible alkyl chain with six carbon atoms. However, these chains are oriented parallel to the surface of the silicate in the aqueous medium of immobilization because of complete immiscibility with it. The distance of the enzyme and the silicate is shorter in GXLG but the epoxy groups can move freely in the aqueous medium of the immobilization reaction and the enzyme is attached to the silicate in a way offering larger mobility. Additionally, an organic medium, like the reaction mixture in the transesterification reaction, solubilizes better the linker with longer than with shorter chain length while the aqueous medium behaves oppositely corroborating our results.

3.3. Degradation studies

The results presented in the previous section proved the attachment of the enzyme to the surface of the silicate and they also showed that it retained its activity after immobilization in reactions taking place in suspension. The final question is if any reaction takes place when neither the enzyme nor the reactant are dissolved in the reaction medium as in the case of a supported enzyme embedded in a polymer. Self-degradable biopolymers were prepared and studied earlier in which an entrapped or physically adsorbed enzyme was added to PCL. In the present study, the degradation of PCL was followed in the presence of immobilized enzymes (*l*PS-GXLG and *l*PS-GAXLG) prepared at room temperature as they

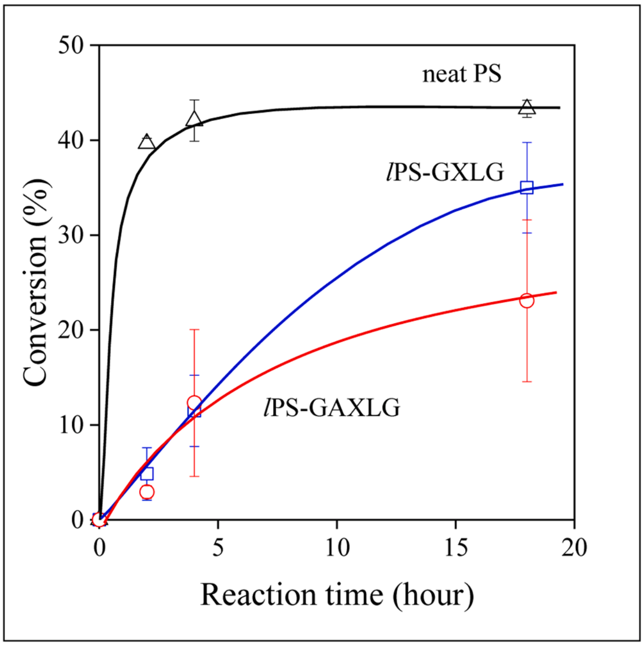


Fig. 6. Conversion achieved in the hydrolysis model reaction (Scheme 2b) as a function of time for neat PS (△), GXLG (□) and GAXLG (◯) immobilized at ambient temperature.

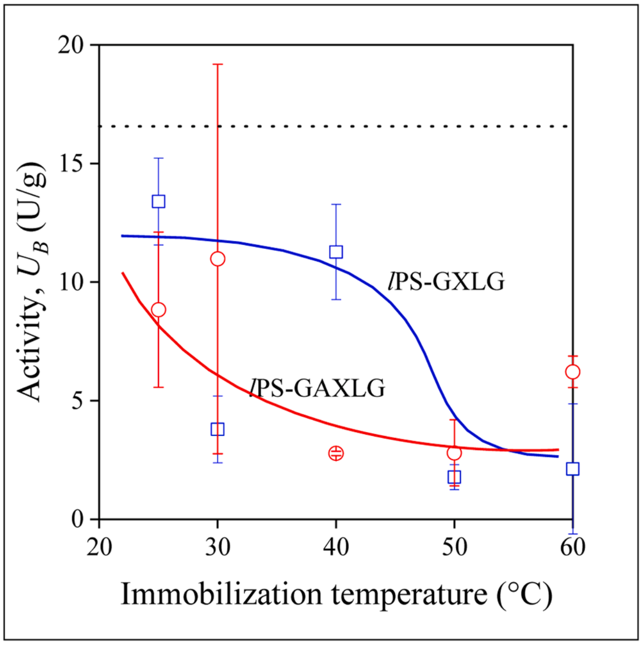


Fig. 7. Enzymatic activity of the lipase immobilized on GXLG (□) and GAXLG (◯) at different immobilization temperatures in the hydrolysis model reaction (Scheme 2b). The horizontal dashed curve is the activity of the neat enzyme.

had the largest enzymatic activity in both model reactions. Composites with different carrier concentrations (2.5, 5, 10 vol%) were prepared and their degradation in aqueous buffer was followed gravimetrically and by UV-Vis spectroscopy [18]. Fig. 8 presents the weight loss of the PCL samples as a function of time, while the absorbance values at 203 nm (proportional to the concentration of the degradation product) recorded on the degradation medium are shown in Fig. 9. According to the results, the enzyme retained its activity during the preparation of PCL composites and the weight loss exceeded 60 % in the case of the sample containing 10 vol% *l*PS-GAXLG. The rate of degradation as well as the amount of the degraded polymer depend on the type of the linker and on the concentration of the immobilized enzyme. Increasing enzyme concentration leads to increasing degradation rate, larger weight loss; and GAXLG has better catalytic activity than GXLG.

The results of the degradation kinetic study were evaluated quantitatively using the model developed earlier for heterogeneous enzyme reactions (Eq. 2)

$$m(t) = v_d\, \tau\, [1 - \exp\,(-t\,/\,\tau)] \qquad (2)$$

in which the three parameters all have real physical meaning; v_d gives the initial rate of degradation at zero time, τ is a time constant proportional to the denaturation rate of the enzyme, and the pre-exponential term $A = v_d\tau$ equals the loss of mass at infinite time, i.e. the amount of polymer degraded by the enzyme. The advantage of the model, unlike most models used for the kinetic analysis of enzymatic reactions, is that it takes into account the denaturation of the enzyme inevitably occurring at longer times or any other factors hindering or stopping degradation. Naturally, the model can be used not only for the analysis of mass loss, but also for any other quantity characterizing the degradation of aliphatic polyesters, i.e. the absorbance of the solution containing the degradation product. The model was fitted to the experimental results (weight loss, absorbance) and the calculated parameters are collected in Table 2.

The results obtained from the two independent methods differ somewhat from each other due to their different reliability, but they clearly show that enzymatic degradation stops after approximately one day as τ is smaller than 38 h for each composition. The comparison of the τ values of GXLG and GAXLG, does not show any difference meaning that the process, which stops the degradation, is similar for the two modification methods. Initial degradation rate is larger in the case of the GAXLG than for the GXLG samples. The same τ values and larger initial degradation rates result in larger extent of degradation in the case of GAXLG.

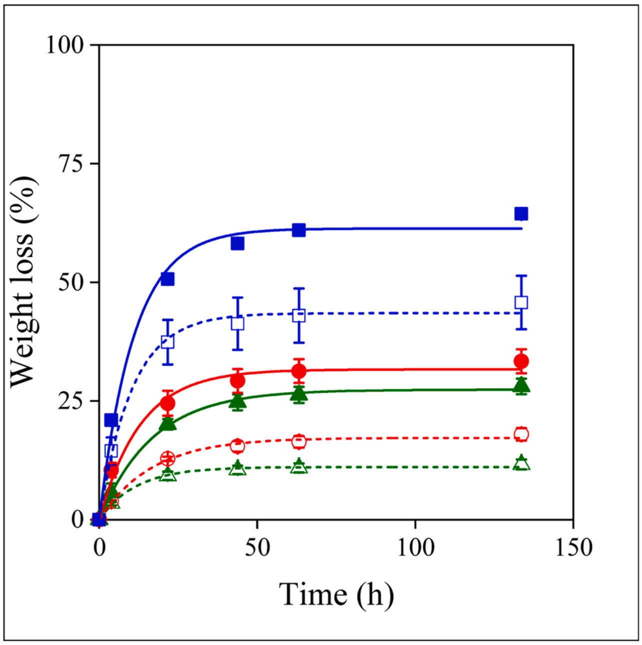


Fig. 8. Weight loss of self-degradable PCL plotted as a function of time at different type (GXLG: open, GAXLG: solid symbols) and amount 2.5 vol% (△,▲), 5 vol% (◯,●), 10 vol% (□,■) of the immobilized enzyme.

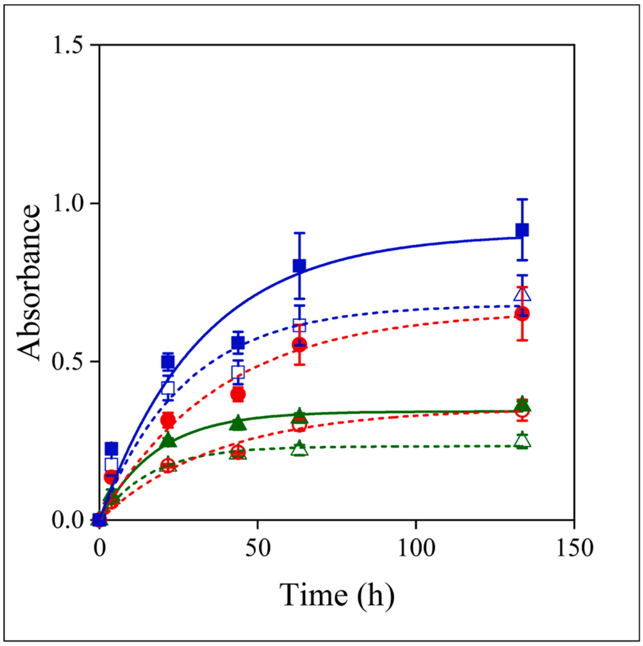


Fig. 9. Absorbance measured at 203 nm as a function of time in the solution of self-degradable PCL at different type (GXLG: open, GAXLG: solid symbols) and amount of immobilized enzyme content 2.5 vol% (△,▲), 5 vol% (◯,●), 10 vol %(□,■); (absorbance values are corrected by dilution and the initial weight of the samples）.

Table 2
Kinetic parameters of the degradation of PCL and the denaturation of the enzyme determined by the proposed model (see Eq. 2)

Immobilized enzyme		Weight loss			Absorbance		
Type	Amount (vol %)	v_d (%/h)	τ (h)	A (%)	v_d (1/h)	τ (h)	A
GXLG	2.5	0.96	11.55	11.07	0.011	15.95	0.23
	5.0	1.04	16.52	17.23	0.009	37.38	0.35
	10.0	4.19	10.37	43.50	0.025	26.90	0.68
GAXLG	2.5	1.62	16.94	27.37	0.019	17.80	0.34
	5.0	2.44	12.98	31.64	0.017	37.75	0.66
	10.0	5.58	10.99	61.33	0.028	32.11	0.91

4. Conclusions

Lipase from *Burkholderia cepacia* was successfully linked covalently to the surface of Laponite® XLG at different temperatures after it has been activated with two different glycidoxy moieties. Functional groups have formed on the surface of the silicate by the activation reaction with silanes and 1,6-hexanediol diglycidyl ether, but just a limited number of the epoxide groups are available for enzyme molecules, because most of the silane compounds are located among the silicate layers. Two model reactions proved the successful immobilization of the enzyme, trans-esterification and hydrolysis. The enzyme retained its activity even after immobilization and the supported enzyme prepared at room temperature had the best catalytic performance; its activity exceeded that of the neat enzyme. The covalently immobilized enzyme efficiently catalyzed the degradation of PCL. Larger than 60 wt% weight loss was achieved during degradation depending on the concentration of the immobilized enzyme. Samples activated in the two-step method have better catalytic performance in polymer degradation than those prepared by the one-step approach.

CRediT authorship contribution statement

Nóra Hegyesi: Writing – original draft, Project administration, Methodology, Investigation, Data curation. **Diána Balogh-Weiser:** Resources, Investigation. **Béla Pukánszky:** Writing – review & editing, Writing – original draft, Supervision, Resources, Conceptualization.

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.

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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.2024.111003.

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