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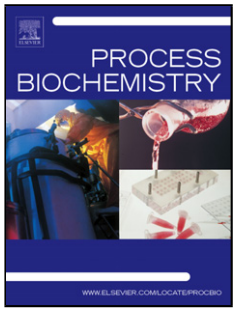

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Enzyme-catalyzed functionalization of poly(L-lactic acid) for drug delivery applications

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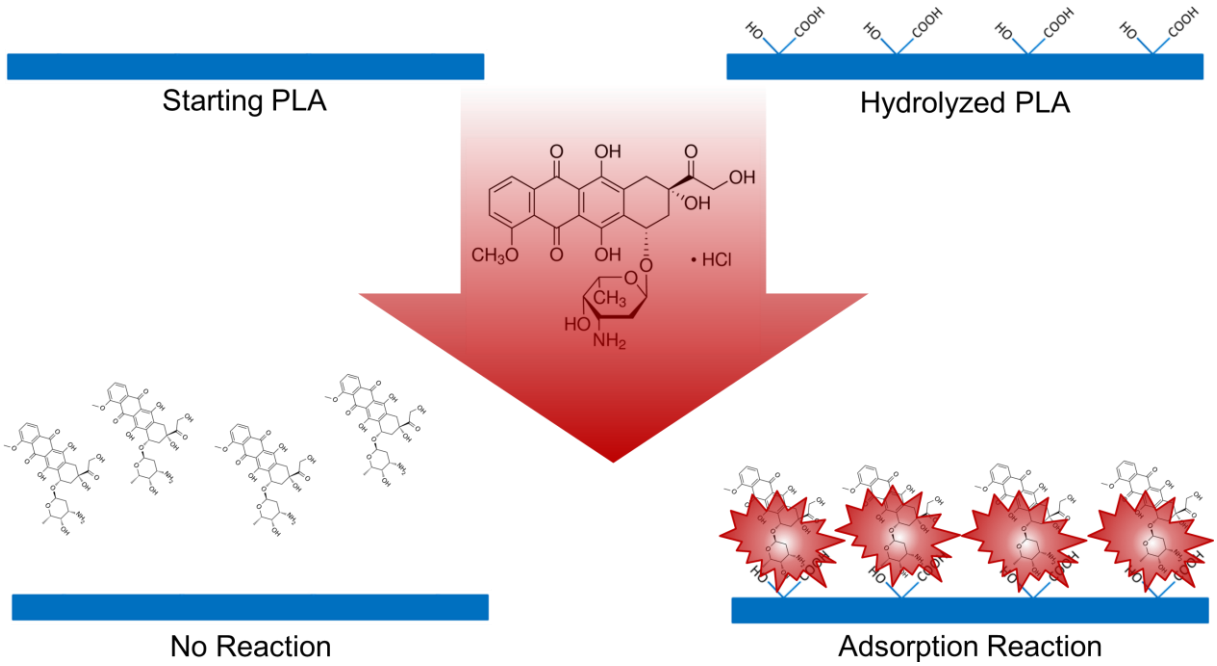
Graphical Abstract



Graphical abstract: enzymatic, environmentally-friendly method for the surface functionalization of PLA films without affecting bulk properties. Onto the enzymatically activated surface doxorubicin, a chemotherapeutic drug, was coupled and adsorption and release kinetics investigated.

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Highlights

- Surface enzymatic functionalization of PLA films was successfully achieved

- Doxorubicin loading and release studies were performed and optimized

- An electrostatic interaction-dependent release of the drug was observed

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Keywords: poly(lactic acid) (PLA); doxorubicin; enzymatic hydrolysis; *Humicola insolens* cutinase (HiC); drug delivery; controlled release.

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Abstract

Polymer-based drug delivery systems are attracting interest for biomedical and, in particular, oncology-related applications due to interesting characteristics in terms of prolonged drug release. In this study, we investigated a new poly(lactic acid) (PLA)-based drug delivery system in which the cationic chemotherapeutic drug doxorubicin was adsorbed via ionic interactions. PLA, a polyester already widely used for biomedical applications due to its biocompatibility and quick assimilation, was initially activated by mild enzymatic surface hydrolysis with cutinase, generating new carboxylic and hydroxyl groups without affecting the bulk properties of the PLA. After the enzyme activation of PLA, the M_n remained almost unchanged, 182 kDa versus 188 kDa for untreated PLA measured by gel permeation chromatography. In contrast, chemical hydrolysis substantially degraded the PLA films as indicated by a decrease of M_n from 188 kDa to 18 kDa. Surface imaging using Scanning Electron Microscopy revealed an increase of granular porosity on the surface upon enzymatic activations while Atomic Force Microscopy showed an increase of the surface roughness from 50 to 170 nm. The hydrophilicity of the enzymatically activated films dramatically increased, as demonstrated by the decrease of the Water Contact Angle from 50° to less than 20°. The negative charges generated on the PLA films was exploited for loading with positively charged doxorubicin; with increasing extent of enzymatic hydrolysis a higher amount of surface functional groups were generated. Desorption studies indicated that the release of doxorubicin from the PLA surface depended on the ionic strength of the medium, thus confirming the ionic nature of the interactions.

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Introduction

Poly(lactic acid) (PLA) is a thermoplastic, renewable, sustainable, FDA approved and biocompatible aliphatic polyester that has been extensively investigated over the last decades [1, 2]. Among several applications such as in food packaging [3] or textiles [4], the development of PLA-based biomedical devices [5] has gained increased interest due to the biocompatibility of this polymer. In the body, PLA degradation leads to lactic acid, an α-hydroxy acid already present in human metabolism [6]. For this reason, PLA is a useful material in clinical practice[7] for tissue engineering, surgery, orthopedic devices and drug delivery. Despite these excellent positive features, drawbacks such as brittleness and chemical inertness limit applications of PLA.

To overcome such limitations, co-polymers of PLA with poly(ethylene oxide) (PEO) and poly(glycolic acid) (PGA) were developed. PLA-PEO-PLA triblock co-polymers demonstrated positive mechanical properties for stent [8] and hydrogel [9] applications while PLA-PGA mixtures displayed good biocompatibility when used in orthopedic implants [10]. The formulations of co-polymers with poly(ethylene glycol) (PEG) and poly(caprolactone) (PCL) were also reported as well as blends with non-polyester based polymers such as dextran and chitosan [11]. On the basis of PLA features, there is growing interest for the applications of various PLA forms, namely copolymers [12] nanoparticles [13, 14] or microspheres [15] for drug delivery. The use of an efficient delivery system enhances the range of drug effectiveness as well as decreased potential system toxicity. Reducing the undesired side effects of drugs on healthy organs is essential in the case of doxorubicin, which besides noteworthy anticancer action and FDA approval [16], still its major toxic side effects, affecting heart, brain, kidneys and liver, with cardiotoxicity as major risk for patients [17]. To limit these side effects, various polymer-based drug delivery systems such as

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liposomes [18], PEGylated liposomes [19, 20], films [21], vesicles [22], hydrogels [23-25], nanoparticles [26-28] and combinations of these delivery strategies [29] were investigated. Nanoparticles and hydrogels were promising delivery systems, however further validation in tumor models is needed in order to evaluate the effectiveness and the delivery range [17]. In any case, improvement of the chemotherapeutics delivery systems for more stable and better applicable devices is still needed.

The lack of reactive groups on PLA suitable for further functionalization hinders some of the utility. Attempts to enhance functionality by introducing thiol groups on nanoparticles [30], corona discharge [31], photografting [32], coating with extracellular matrix proteins and plasma treatment [11] are only a few of the investigated treatments for PLA surface functionalization. Moreover, recent reports indicate how hydrolytic enzymes such as lipases [33, 34] and cutinases, [35-37] belonging to the serine hydrolases superfamily, are useful catalysts to activate the surface of PLA. Enzymatic, mild PLA hydrolysis of the outer polymer layers generates new carboxylic and hydroxyl groups on the surface, in a much more controlled way than conventional alkaline hydrolysis, which can lead to complete polymer destruction [36].

In the present work, we investigated the potential for enzymatic generation of carboxylic and hydroxyl groups on PLA to improve loading with the chemotherapeutic drug doxorubicin via ionic interactions. This drug-loaded Janus-faced PLA with an enzymatically-activated surface and unaltered bulk polymer properties opens new perspectives for drug loading and delivery.

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Materials and methods

Chemicals, reagents and enzymes

Poly(L-lactic acid) PLA films, thickness 50 µm, were purchased from Goodfellow (Cambridge, UK). Doxorubicin hydrochloride salt (purity >99%) was purchased from LC Laboratories (Woburn, USA). All other chemicals and solvents were purchased from Sigma-Aldrich at reagent grade, and used without further purification if not otherwise specified. *Humicola insolens* cutinase (HiC) was kindly provided by Novozymes (Beijing, China), concentration 11.2 mg mL^{-1} and was purified via ultrafiltration prior to use as previously reported [36].

Esterase activity assay and Protein quantification

Esterase activity was measured at 21˚C using p-nitrophenyl butyrate (PNPB) as a substrate as previously reported [36]. The activity was calculated in units (U), where 1 U is defined as the amount of enzyme required to hydrolyze 1 µmol of substrate per minute under the given assay conditions. The protein concentration of the enzyme solution was determined with the BioRad protein assay (Bio-Rad Laboratories GmbH, Vienna, Austria) as previously reported [36].

PLA Hydrolysis

Prior to the treatment, PLA films were cut into 10×10 mm pieces and washed in three consecutive steps for 30 min at 37 °C and 130 rpm. In the first step, a solution of 5 g L^{-1} Triton-X100 was used in the second step a 100 mM Na_2CO_3 solution was used, followed by double distilled water (ddH_2O). Then 5 µM of *Humicola insolens* cutinase was diluted in 100 mM Tris-HCl buffer pH 7 and used for PLA hydrolysis. Chemical hydrolysis was performed using different concentrations of NaOH. Incubations were performed at 130 rpm and 37 °C for the enzymatic treatment and at 21 °C and 130 rpm for the alkaline hydrolysis. Hydrolysis products were quantified by HPLC using refractive index detection (1100 series, Agilent Technologies, Palo Alto,

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CA) equipped with an ICSep-ION-300 column (Transgenomic Organic, San Jose, CA) of 300 mm by 7.8 mm and 7 µm particle diameter. Column temperature was maintained at 45 °C. Samples (40 µL) were injected and separated by isocratic elution for 40 min at 0.325 mL min^{-1} in 0.005 M H_2SO_4 as the mobile phase. As control, PLA was incubated with a buffer solution (in the case of enzymatic hydrolysis) or mQH_2O (in the case of alkaline hydrolysis).

Doxorubicin loading

After enzymatic hydrolysis, PLA films were incubated in 3 mL doxorubicin solutions at various concentrations (2.5, 25, 50 and 100 µM) at 25°C and 130 rpm in an orbital shaker at different times (1, 3 and 6 days) in order to determine the best conditions for the drug adsorption onto the activated polymer surface. After the incubation, PLA films were washed three times with mQH_2O and immediately incubated in the desorption media. All experiments were performed in triplicates.

***In vitro* Doxorubicin release**

The doxorubicin-loaded PLA films were incubated with mQH_2O or 1X PBS buffer pH 7.4 (in order to mimic the physiological environment) and the kinetic of the drug-polymer surface desorption was monitored over time in 3 mL media volume via photometric analysis. All experiments were performed in triplicates.

Gel Permeation Chromatography (GPC)

GPC samples were prepared and analyzed as previously reported [36] using a Agilent Technologies HPLC System (Agilent Technologies 1260 Infinity) connected to a 17369 6.0 mm ID x 40 mm L HHR-H, 5 µm Guard column and a 18055 7.8 mm ID x 300 mm L GMHHR-N, 5 µm TSKgel liquid chromatography column (Tosoh Bioscience, Tessenderlo, Belgium) using THF (250 ppm BHT as inhibitor) as eluent (at a flow rate of 1 mL min^{-1}). An Agilent Technologies G1362A refractive index

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detector was employed for detection. The molecular weights of PLA were calculated using linear polystyrene calibration standards (400-2,000,000 Da).

Hydrophobicity measurements

Hydrophobicity was measured via water contact angle analysis (WCA). WCA of the PLA films was measured before and after exposure to the enzymatic treatments (and control reactions). Polymer films were analyzed with a Drop Shape Analysis System DSA 100 (Kruss GmbH) using ddH_2O as reference liquid with a drop size of 2 µL and a deposition speed of 100 µL min^{-1}. Water contact angles were measured via the static sessile drop method after 5 s. Data were obtained from the averages of measurements taken from three different points of three different sample surfaces.

Fourier transform infrared spectroscopy analysis (FT-IR)

The surfaces of PLA films before and after hydrolysis and doxorubicin loading were characterized using a Perkin Elmer Spectrum 100 FT-IR Spectrometer. Spectra were collected at a resolution of 4 cm^{-1} for 8 scans. The bands were assigned as follows: 3,600-3,100 cm^{-1} $v(NH_2)$, 3,600-2,900 cm^{-1} v(OH), 2,996-2,945 cm^{-1} $v(CH_2)$ and $v(CH_3)$, 1,748 cm^{-1} v(C=O), single band 1,620 cm^{-1} $\delta(NH_2)$, single band 1590 cm^{-1} v(C=C), 1,452 cm^{-1} $\delta(CH_2)$, 1,299 cm^{-1} v(C-O-C), 1,128 cm^{-1} v(C-O), 957 cm^{-1} v(C-O-C).

Confocal Laser Scanning Microscopy (CLSM)

Images of PLA films with different concentrations of adsorbed doxorubicin (2.5, 25, 50 and 100 µM) at different times (24, 48 and 72 h) were collected by Olympus FV1000 microscope (excitation wavelength 488-515 nm; emission wavelength 530-590 nm) both with 60X water immersion lens for detailed images and 20X for fluorescence quantifications. Quantifications of the surface-distributed signal intensities were calculated applying the integrated density method with background

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subtraction. Each value is an average of the calculations from three different areas of the related image.

Scanning Electron Microscopy (SEM)

PLA films morphology was qualitatively assessed through SEM. Control PLA (without any enzymatic attack) and enzymatically modified PLAs (after 24, 48 and 72 h of enzymatic hydrolysis) were surface characterized. All SEM images were acquired collecting secondary electrons on a Gemini SUPRA 40 SEM (Carl Zeiss NTS GmbH, Oberkochen, Germany) working at 2 kV acceleration voltage. Prior to SEM characterization samples were gold metalized on a metal sputter coater.

Atomic Force Microscopy (AFM)

For the AFM characterization, PLA films were cut in 5×5 mm^2 pieces and mounted on magnetic plates using double-side adhesive tape. AFM was used in dynamic mode at room temperature in air using a commercial instrument (Solver Pro, NT-MDT, RU). Silicon tips (HA_FM ETALON series probes from NT-MDT, RU) with a typical force constant of 6 nN nm^{-1} and a resonance frequency of about 114 kHz were employed. Topographic height and phase images were recorded at 512×512 pixels at a scan rate of 1 Hz. Image processing, profile extrapolation, roughness analysis and surface areas evaluation were all performed using Gwyddion freeware AFM analysis software, version 2.40 [38]. Surface roughness was computed starting from AFM three-dimensional surface reconstructions as the root-mean-square (RMS) value of the height irregularities of AFM images and indicated as R_{rms}. The ratio between the surface area and the projected surface area, was computed from AFM images by simple triangulation and correlated to roughness values and indicated as surface variation ΔS%.

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Results and Discussion

Hydrolysis of PLA films

In order to enhance electrostatic binding of doxorubicin the surface of PLA was "activated" based on limited enzymatic or chemical hydrolysis [36]. According to GPC analysis (Fig. 1), alkali-based hydrolysis of the films led to an unacceptable reduction of the polymer M_n and M_w after only 10 min of reaction time indicating degradation of the polymer bulk. In addition, the strong alkaline treatment will cause difficulties related to removal of NaOH after the process [11].

Enzymatic hydrolysis of PLA films was conducted using *Humicola insolens* cutinase (HiC) [35] according to the method we have recently reported restricting hydrolysis to the PLA surface without affecting the bulk properties of the polymeric films, as demonstrated from the GPC data [36].

Surface characterization of PLA film cross sections (pristine, 72 h control treatment without enzyme, 24, 48 and 72 h of enzymatic treatment, respectively) via scanning electron microscopy (SEM) qualitatively revealed the presence of a granular porosity localized at sample surface (right side of SEM images in Fig. 2A) that increased in both amount and depth with increasing time of the enzyme treatment. Conversely, the control PLA sample showed unaltered bulk properties when compared to pristine PLA. To quantify this surface modification, we reconstructed the three-dimensional surface of PLA samples using AFM evaluating the variation between the exposed sample surface and the protected surface (ΔS%). For pristine and control PLA the former was larger than the latter of about 0.38%. Enzymatic treatment induced an increase of ΔS% of about 13.2% for 24 h treatment, 15.8% for 48 h treatment and 16.4% for the 72 h treatment (Fig. 2B). Surface roughness and function of time were evaluated from AFM images. In particular, 24 h enzymatic reaction on PLA led to a surface roughness R_{rms} of 98.3 nm compared to 50.5 nm characterizing the pristine

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PLA surface. The 48 and 72 h treatments lead to 141.5 nm and 171.0 nm of R_{rms} values, respectively (Fig. 2C). The hydrolysis process induced in the pristine PLA surface an increase of roughness of about 196%, 283% and 342%, respectively, for 24, 48 and 72 h of incubation. This is a clear indication, as pointed out by the increase in ΔS%, of a reduction in sample's surface homogeneity and an increase of the exposed surface area as function of reaction time. This result should improve the ability of PLA to trap or bind to molecules at the surface (Fig. 4). In addition to an increase in roughness, enzymatic surface hydrolysis led to strong increases in surface hydrophilicity (Fig. 2D). Without compromising the bulk properties, a decrease of water contact angle decrement of around 40° was measured.

In accordance with previous reports [2] FT-IR analysis was used for the characterization of the PLA surface clearly demonstrating the formation of new hydroxyl and carboxylic groups on the polymer surface (see ESI, Fig. 1s) responsible for the marked hydrophilicity increase noticed via WCA analysis (see Fig. 2).

In conclusion, the present results provide evidence for the generation of hydroxyl and carboxylic groups on the surface of PLA films with concomitant increase of hydrophilicity upon limited enzymatic hydrolysis of ester bonds. In contrast to alkaline hydrolysis, GPC data confirmed that the enzymatic treatment allowed to fully preserve the bulk properties of PLA. Hence, this enzymatic treatment alone could improve PLA properties for many applications ranging from medical to textiles. For example, cell viability or moisture regain of textiles can be improved without use of chemicals and loss of beneficial bulk properties [35].

Doxorubicin loading studies

The effect of enzymatic PLA surface "activation" on drug loading capacities using doxorubicin hydrochloride salt (Doxo) was investigated. The release of Doxo was previously investigated from silk films where the effect of negatively charged

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chemical modification on release properties was investigated [21]. Photometric quantification of Doxo adsorbed on the PLA films (Fig. 3) demonstrated that the polar and charged groups generated on the PLA film surface were a necessary pre-requisite for establishing electrostatic interactions between the polymer surface and the drug. The photometric analysis vas performed using a portable ColorLite photometer that enabled the evaluation of the Lab color space parameters of each sample. All measurements were conducted on three independent samples.

Increasing the Doxo solution concentration while keeping the incubation time of enzymatic surface activation constant resulted in increased drug adsorption on the polymer surface (Fig. 3). Fluorescence-based quantification of the loaded drug was performed via CLSM analysis (Fig. 4). The obtained results are consistent with the photometric method and denote the pre-activation hydrolysis step of 72 h as the optimal condition for the drug loading.

After the hydrolytic step, the polymer was incubated with Doxo for 1, 3 and 6 days. Figure 4 illustrates that the highest amount of PLA-bound Doxo was obtained after 3-days incubation. Additionally, FT-IR spectroscopy confirms the adsorption of the drug on the activated polymer surface (Fig. 5).

FT-IR analysis demonstrated that the cationic chemotherapeutic drug Doxo was effectively bound to PLA via electrostatic interactions onto after limited enzymatic surface hydrolysis. The highest loading was achieved using PLA films (1.0x1.0 cm) obtained after three days of hydrolytic pre-treatment (thickness = 50 µm, M_n= 26,493, M_w= 188,104, PD= 7.142.) and incubated for three days in a 100 µM Doxo solution at pH 7.0. Hence, these parameters were used for drug release studies.

Doxo release studies

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After a quick wash (10 min, 130 rpm, 21°C) of the Doxo loaded PLA films with mQH_2O, we performed *in vitro* release studies incubating the enzymatically activated and drug-loaded PLA with mQH_2O and 1X PBS pH 7.4 buffer, respectively.

Doxo released faster from the PLA films when PBS was used when compared to mQH_2O (Fig 6). This is most likely due to displacement of doxorubicin with charged ions contained in the PBS not present in mQH_2O. After 24 h, a plateau in doxorubicin release was observed both for water and PBS buffer. The above presented kinetic data are in accordance with the release of Doxo from carboxylate silk films previously reported [21]. Additionally, an estimation of the remaining bound Doxo on the PLA film was performed. In agreement with data shown in Figure 6, a higher percentage of Doxo remained bound to the PLA when mQH_2O was used as desorption medium, while using 1X PBS pH 7.4 lead to a higher release of the drug from PLA (see ESI, Fig. S2).

This novel PLA-based Doxo release system, would exploit already proven doxo therapeutic benefits in nanosystems like liposomal formulations used for the treatment of recurrent breast cancer [16], while overcoming the limitations of current materials & formulations. For example, liposomes showed signs of leakage and easy recognition/removal from the circulatory system via the reticuloendothelial system [39, 40]. For this reason, so called stealth liposomes (liposomes covered with PEG) were developed in order to prolong their circulation time [41] and plasma half-life [42]. In combination with nano targeting delivery systems, like lyso-thermosensititve liposomes, doxorubicin´s systemic toxicity profile is improved [43]. Recently chitosan/dipotassium orthophosphate hydrogels showed to be promising for the local treatment of bone tumors [42] and dextran-graft-PEI nanoparticles were used on osteosarcoma cells [44, 45].

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Conclusions

A mild and selective enzymatic method for the functionalization of the surface of PLA films without altering its bulk properties is described. By tuning the extent of enzymatic hydrolysis it is possible to tune the degree of surface hydrophilicity and roughness to PLA films. Due to the introduction of carboxylic and hydroxyl groups on PLA surface, the binding of the chemotherapeutic drug doxorubicin by electrostatic interactions was enhanced while release of the chemotherapeutic drug doxorubicin is driven by electrostatic interactions. The PLA-Doxo release system is of interest for potential applications in patients affected by osteosarcoma or similar bone cancers where the insertion of a metal prosthesis is needed. Biocompatible materials endowed with tumor suppressive activity, such as the reported Doxo-loaded PLA, could be exploited for coating the artificial implants and improve both the anticancer effect and the acceptance of the inserted device by the organism. More generally, the enzymatically functionalized PLA deserves further attention and investigations for exploring their effectiveness in formulation of different drugs for selected applications.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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Figure 1. Hydrolysis of PLA films by 5 µM *Humicola insolens* cutinase for 72 h and 0.1 M NaOH (alkali-based) for different incubation times.

Figure 2. Characterization of PLA film surface before and after enzymatic hydrolysis (Hydro) for different time intervals when compared to a control (CTRL) via A) SEM imaging, B/C) AFM analysis of exposed surface area and D) WCA. Data represent the mean ± S.D. from three different samples and is >5% where not shown in the graphics.

Figure 3. Photometric analysis of Doxo adsorbed on PLA films pre-activated through 24 (top) and 72 h (bottom) enzymatic hydrolysis. Grey bars: pre-activated PLA films; white bars: control reactions. Data represent the mean ± S.D. from three different samples.

Figure 4. Quantification of the PLA-bound Doxo via CLSM after different enzymatic pre-hydrolysis treatments. The selected Doxo concentration was 100 µM. Data represent the mean ± S.D. from three different samples and is >5% where not shown in the graphics.

Figure 5. FT-IR spectroscopy of the PLA activated film coupled with various concentrations (2.5, 25, 50 and 100 µg mL^{-1}) of Doxo for 3 days.

Figure 6. Doxo release studies from enzymatically activated PLA in PBS (grey bars) and in mQH_2O (white bars). Data represent the mean ± S.D. from three different samples.

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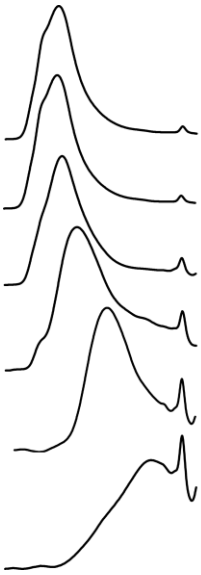

Treatment	M_n	M_w
Starting PLA	26493	188104
72h Enzymatic Hydrolysis	25874	181826
10min	18662	128248
20min	9466	51007
30min	6738	37827
60min	3984	18178

Figure 1

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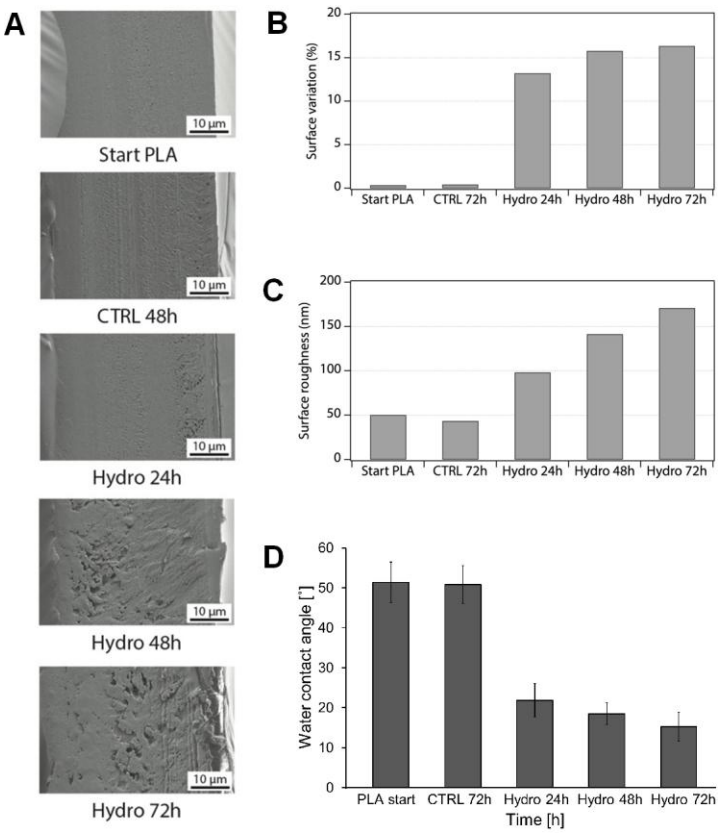

Figure 2

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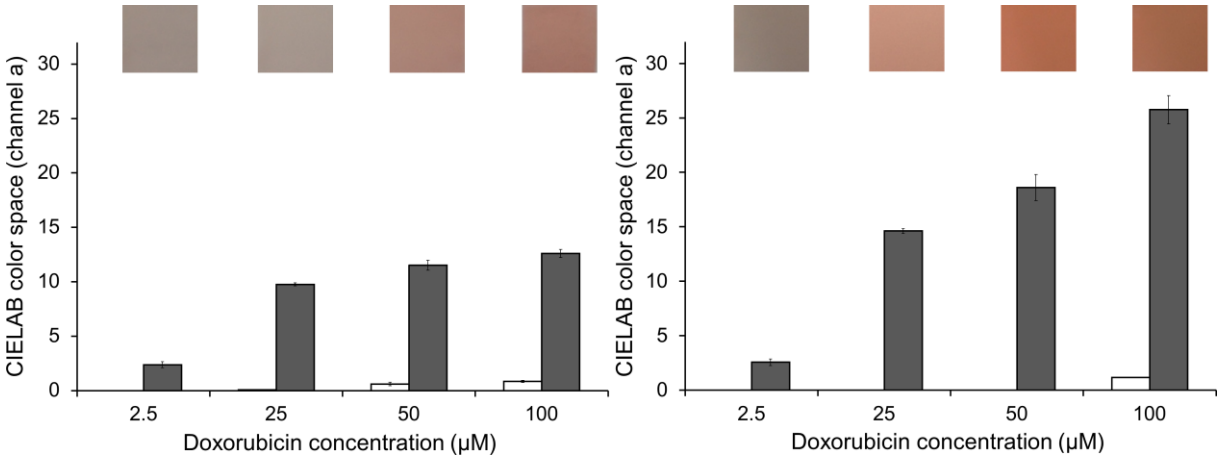

Figure 3

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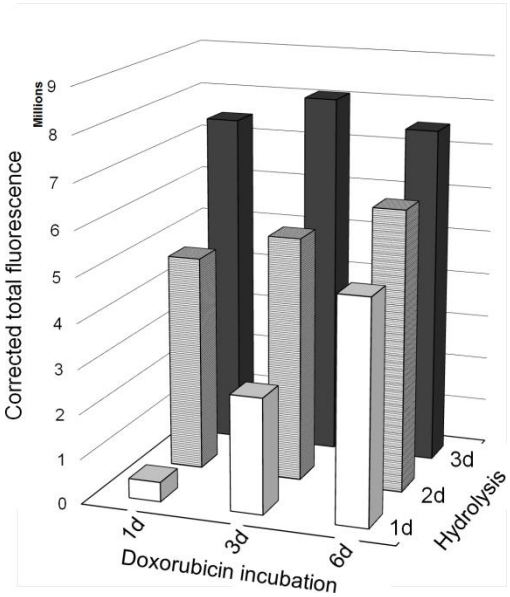

Figure 4

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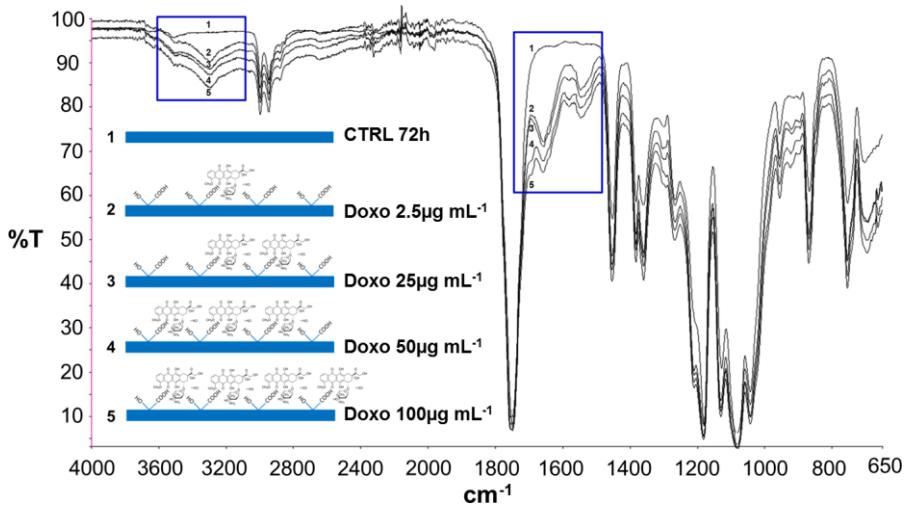

Figure 5

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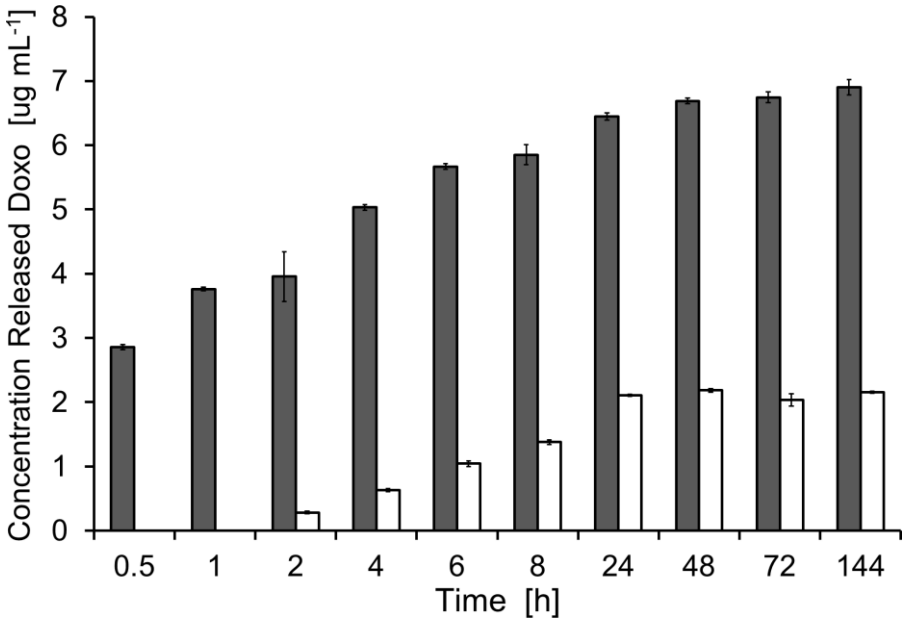

Figure 6