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Communication

Enzymatic Degradation of Oligo(ε-caprolactone)s End-Capped with Phenylboronic Acid Derivatives at the Air-Water Interface [1]

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The influence of terminal functionalization of oligo(ε-caprolactone)s (OCL) with phenylboronic acid pinacol ester or phenylboronic acid on the enzymatic degradation behaviour at the air-water interface is investigated by the Langmuir monolayer degradation technique (LMD). While the unsubstituted OCL immediately degrades after injection of the enzyme lipase from *Pseudomonas cepacia*, enzyme molecules are incorporated into the films based on end-capped OCL before degradation. This incorporation of enzymes does not inhibit or suppress the film degradation, but retards it significantly. A specific binding of lipase to the polymer monolayer allows studying the enzymatic activity of bound proteins and the influence on the degradation process. The functionalization of a macromolecule with phenyl boronic acid groups is an approach to investigate their interactions with diol containing biomolecules like sugars and to monitor their specified impact on the enzymatic degradation behavior at the air-water interface.

[1] **Supporting Information** is available online from the Wiley Online Library or from the author.

1. Introduction

For the introduction of new polymers in medical or pharmaceutical applications a detailed knowledge of their stability in physiological environment or of their long-term degradation behavior is essential. The prominent challenge for developing innovative biomaterials is to avoid undesirable foreign body reactions like immunological reactions, and to ensure that potential degradation products are non-toxic.[1] The biocompatibility of a biomaterial is mainly determined by interface triggered interactions with the biological environment. Investigations of ultrathin polymer films at the air-water interface allow studying interactions between polymer monolayers and biomolecules like enzymes in order to elucidate adsorption processes of enzymes at biomaterial surfaces.[2] Additionally, hydrolytic and enzymatic degradation of polymer monolayers can be recorded as a function of type and pH of the degradation medium by Langmuir monolayer degradation (LMD) technique.[3-10] In comparison to bulk degradation methods, diffusion and transport phenomena can be neglected when it is ensured that the whole polymer and its cleavable bonds are in direct contact with the aqueous media and no crystallisation or aggregation within the polymer layers occur. Furthermore, LMD studies are not as time consuming as bulk degradation experiments.

To elucidate the influence of proteins on the degradation of a polymeric layer at the air-water interface studies are needed how proteins interact with the polymer surface and how conformational changes of the proteins influence their degradation activities. For LMD investigations of specific interactions between a polymer film and proteins an advanced polymer model system is required. We hypothesize that the functionalization of polymers such as polyesters with phenyl boronic acid or its derivatives opens a way for such investigations. Phenyl boronic acid derivatives have not only specific applications in organic chemistry, but also as therapeutic agents, drug delivery systems or enzyme inhibitors.[11-13] Boron containing reagents are often used in the organic chemistry because of the structure of

boron as electron-pair acceptor. In aqueous systems there is always an pH-dependent equilibrium between the neutral trigonal sp^2 boronic acid ($R\text{-}B(OH)_2$; R = alkyl, aryl), acting as a Lewis acid which can easily be converted to an anionic tetrahedral sp^3 boronic acid compound.[14] Whereas the anionic form leads very often to hydrophilic polymers, the neutral polymers behave hydrophobic.[11] Those anionic boronate anions are able to covalently bind 1,2- or 1,3-diols like saccharides to form cyclic boronate esters (Scheme S1). The pK_a values of phenylboronic acids (PBA) range from 4.5 - 8.8[15] strongly depending on substituents linked to the phenyl ring and can also be tuned by the addition of other substituents.[11] Polymeric PBA without further substituents have a pK_a in the range of 8 - 9.[16] To obtain stable bonds between the boronic acid and diols under physiological (pH 7.4) or weak acidic conditions, it is necessary to use e.g. saccharides in a high concentration to enhance the acidity of the phenylboronic acid.[17, 18] These specific interactions of boronic acid derivatives with hydroxyl groups or saccharide units as they appear in many biomolecules or bacteria membranes, for example, may also enable investigations of the impact of biomolecules on the polymer layer's morphology and on its degradation behavior at the air-water interface.

For enzymatic degradation experiments lipase from *Pseudomonas cepacia* is very well established. This lipase contains the catalytic triad consisting of Ser87, His286 and Asp264[19, 20] whereby the nucleophilic Ser87 unit is located at the C-terminal edge of the central β strand.[21, 22] X-ray analyses of different lipases revealed that the nucleophilic hydroxyl function of Ser87 attacks amide as well as ester carbonyl groups and catalyses the hydrolysis of e.g. polyesters.[23] Because of their Lewis acid character boronic acids may also be able to bind nucleophilic Ser87 units.

Therefore, in this work first the ability of monolayers based on phenylboronic acid derivatives end-capped oligo(ε-caprolactone) to interact with *Pseudomonas cepacia* lipase is investigated in comparison to phenyl boronic acid free OCL monolayers. The second aspect is to explore

whether the interactions of OCL functional end-groups with *Pseudomonas cepacia* lipase influence the chain scission mechanism from random cut to chain-end cut. OCL was chosen as a model system, because it does not show any autocatalysed hydrolysis. Furthermore, its compression and crystallization behavior at the air-water interface is known.[24]

2. Experimental Section

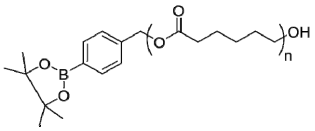
2.1 Materials

4-(Hydroxymethyl)phenylboronic acid pinacol ester, DOWEX-50WX2 ion-exchange resin, oligo(*ε*-caprolactone) (OCL-2K), *p*-toluenesulfonyl chloride, pyridine, sodium azide, copper(I)bromide, D-(+)-glucose, saccharose and phosphate buffered saline (PBS) were purchased from Sigma Aldrich. *ε*-Caprolactone, $Sn(Oct)_2$ and 4-ethynylbenzenboronic acid pinacol ester were purchased from Alfa Aesar. N,N,N',N'',N''-pentamethyldiethylenetriamine was purchased from Merck. DOWEX-50WX2 ion-exchange resin was washed with THF prior to use, all other substances were used without further purification.

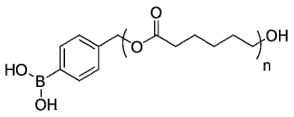
2.2 Synthesis

Boron functionalization was performed as described in literature[25] by ring-opening polymerization of *ε*-caprolactone and 4-methylphenylboronic acid pinacol ester (Bpin) with $Sn(Oct)_2$ as catalyst to obtain Bpin-OCL as a white crystalline solid. 4-Methylphenylboronic acid end-capped OCL (B-OCL) was provided as a white crystalline solid by deprotection of the pinacol ester with DOWEX-50WX2 ion-exchange. For functionalization of both chain ends the hydroxyl end group of the pinacol ester Bpin-OCL was tosylated by the use of pyridine and *p*-toluenesulfonyl chloride followed by a conversion of the tosyl end group by sodium azide. Afterwards the azide end group was converted into a 1,2,3-triazol boronic acid

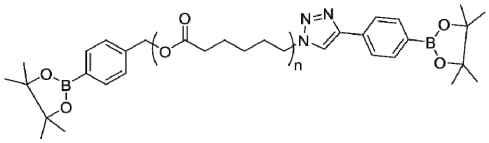
pinacol ester by a copper catalyzed azide-alkyne coupling to obtain OCL double end-capped by Bpin (Bpin-OCL-Bpin) as a white crystalline solid. Finally, the deprotection of the pinacol ester groups was performed as described above to yield B-OCL-B as a white crystalline solid.[25]



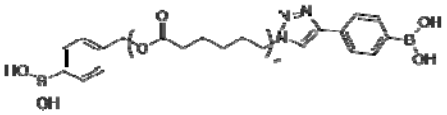
Bpin-OCL



B-OCL



Bpin-OCL-Bpin



B-OCL-B

Bpin-OCL: ^{1}H-NMR ($CDCl_3$, 300MHz): δ (ppm) 7.81 (d, 2H, CH), 7.35 (d, 2H, CH), 5.12 (s, 2H, CH_2), 4.05 (t, $CH_2O(C=O)$), 3.64 (t, CH_2OH), 2.30 (t, $CH_2(C=O)O$), 1.63 (m, 4H, CH_2), 1.37 (m, 2H, CH_2). GPC ($CHCl_3$): M_n = 4300 g·mol^{-1}, M_w = 4600 g·mol^{-1}, PDI = 1.08.

B-OCL: ^{1}H-NMR ($CDCl_3$, 300MHz): δ (ppm) 7.77 (d, 2H, CH), 7.35 (d, 2H, CH), 5.12 (s, 2H, CH_2), 4.05 (t, $CH_2O(C=O)$), 3.67 (t, CH_2OH) 2.30 (t, $CH_2(C=O)O$), 1.64 (m, 4H, CH_2), 1.37 (m, 2H, CH_2). GPC ($CHCl_3$): M_n = 2500 g·mol^{-1}, M_w = 2900 g·mol^{-1}, PDI = 1.16.

Bpin-OCL-Bpin: ^{1}H-NMR (acetone-d_6, 300 Hz): δ (ppm) 7.85-7.78 (m, 2H, CH), 7.35 (d, 2H, CH), 5.12 (s, 2H, CH_2), 4.41 (t, CH_2OH), 4.05 (t, $CH_2O(C=O)$), 2.32-2.27 (m, $CH_2(C=O)O$), 1.67-1.60 (m, 4H, CH_2), 1.41-1.33 (m, 2H, CH_2). GPC (THF): M_n = 6300 g·mol^{-1}, M_w = 6400 g·mol^{-1}, PDI = 1.01.

B-OCL-B: ^{1}H-NMR (acetone-d_6, 300 Hz): δ (ppm) 8.40 (d, 1H, CH), 7.94-7.82 (m, 2H, CH), 7.39 (d, 2H, CH), 5.15 (s, 2H, CH_2), 4.49 (t), 4.06 (t, $CH_2O(C=O)$), 2.35-2.30 (m, CH_2 (C=O)O), 1.67-1.61 (m, 4H, CH_2), 1.44-1.35 (m, 2H, CH_2). GPC (THF): M_n = 5600 g·mol^{-1}, M_w = 5800 g·mol^{-1}, PDI = 1.04.

2.3 Methods

^{1}H-NMR data were obtained on a Bruker *Avance* 300 MHz system at 25 °C in $CDCl_3$ (VWR, 99.8 %).

Gel permeation chromatography (GPC) measurements were performed to determine the molecular weight of the polymer using a multidetector GPC equipped with one precolumn, two 300 mm × 8.0 mm linear M columns (Polymer Standards Service GmbH, Mainz, Germany, PSS), an isocratic pump 2080 and an automatic injector AS 2050 (both Jasco, Tokyo, Japan) in $CHCl_3$ at a flow rate of 1.0 mL·min^{-1} and 0.2 wt% toluene as internal standard at 35 °C. The injected samples had a concentration of 1.0 mg·mL^{-1}. An RI detector Shodex RI-101 (Showa Denko, Japan) as well as a viscosimeter SEC-3010 (WGE, Dr. Bures, Dallgow, Germany) were used combined by a split. The molecular weight determination was performed by the help of SEC software WINGPC Unity (Polymer Standards Service GmbH) using a universal calibration with polystyrene standards (M_n = 580 - 975000 g·mol^{-1}, Polymer Standards Service GmbH).

Morphology investigations by Brewster angle microscopy (BAM) were simultaneously performed by an imaging ellipsometer (nanofilm_ep3, Accurion, Göttingen, Germany) during π-*A* isotherm experiments.

Surface pressure-area (π-*A*) isotherms were recorded by a KSV high-compression Langmuir trough system (KSV Nima LTD, Espoo, Finland) with a barrier speed of 10 mm·min^{-1} at a temperature of 22 ± 1 °C. $CHCl_3$ stock solutions (Roth, ≥ 99.0 %) of Bpin-OCL, B-OCL, Bpin-OCL-Bpin, B-OCL-B and OCL-2K at a concentration of 0.25 mg·mL^{-1} and a volume of 50 µL were spread onto the surface of ultrapure water by the use of a Hamilton 250 µL syringe. The surface pressure π was determined using a Wilhelmy film balance after the

solvent has evaporated over a period of 10 min. All Langmuir experiments were performed on ultrapure water (≥ 18.2 MΩ·cm, Milli-Q, Millipore) or solutions of it.

The total area loss $\Delta A(t)$ is defined as the difference between the initially occupied surface area A_0 and the occupied surface area $A(t)$ after a certain degradation time t (Equation 1):

$$\Delta A_{rel}(t) = [A_0 - A(t)] \cdot A_0^{-1} \qquad \text{(Equation 1)}$$

By the use of the dynamic fragmentation model the corrected relative area change $\Delta A_{corr}(t)$ is defined as the generation of water-soluble fragments per unit area (Equation 2):[26-28]

$$\Delta A_{corr}(t) = \Delta A_{rel}(t) \cdot [A_0 \cdot A(t)^{-1}] = [A_0 \cdot A(t)^{-1}] - 1 \qquad \text{(Equation 2)}$$

LMD studies of monolayers were performed on a phosphate buffered saline (PBS) subphase at a pH of 7.4. Solutions of *Pseudomonas cepacia* lipase in PBS at a concentration of 0.25 mg·mL^{-1} and a volume of 5 mL were injected into the subphase, after $CHCl_3$ stock solutions (Roth, ≥ 99.0 %) of Bpin-OCL, B-OCL, Bpin-OCL-Bpin, B-OCL-B or OCL 2K at a concentration of 0.25 mg·mL^{-1} and a volume of 50 µL were spread onto the subphase. The monolayers were compressed to reach a surface pressure $\pi = 7$ mN·m^{-1} which was kept constant afterwards. The change in the mean molecular area per repeating unit (MMA) was monitored over a certain period of time. The schematic experimental set-up is shown in Scheme S2.

3. Results and Discussion

3.1 Langmuir monolayer characterization

To elucidate whether the phenyl boronic acid end groups influence the Langmuir and crystallization behavior of OCL monolayers the corresponding π-*A* isotherms were recorded (Figure 1). The samples are of comparable molecular weight (M_w = 2900 – 6400 g·mol^{-1}) and

thus, differences in film compression and crystallization behavior induced by different molecular weights can be excluded and just rely on the introduction of boronic acid end groups.

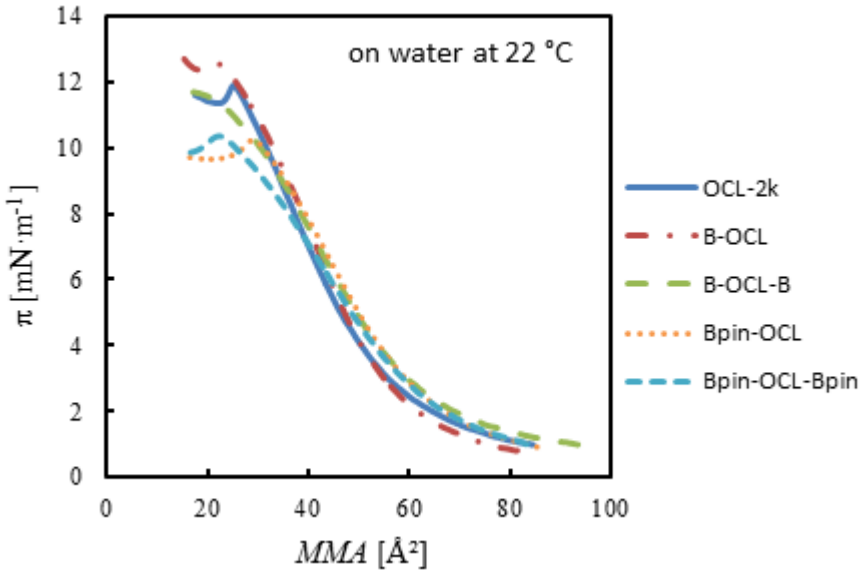


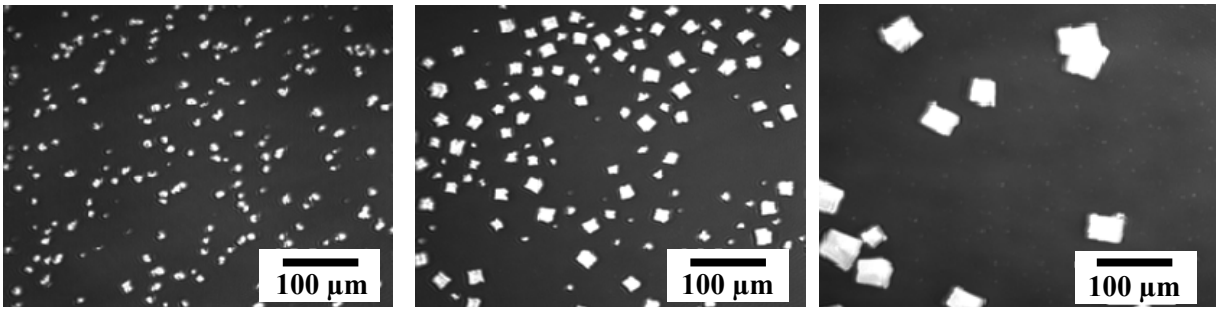
Figure 1. Plot of surface pressure π as a function of *MMA* of Bpin-OCL, B-OCL, Bpin-OCL-Bpin, B-OCL-B and OCL-2K monolayers at the air-water interface at $T = 22$ °C.

Table 1. Inflection point data MMA_i and π_i for Bpin-OCL, B-OCL, Bpin-OCL-Bpin, B-OCL-B and OCL-2K Langmuir films at 22 °C

Sample ID	MMA_i [Å^2]	π_i [mN·m^{-1}]
OCL-2K	36.0	8.4
B-OCL	37.5	8.1
B-OCL-B	42.0	7.1
Bpin-OCL	47.0	6.0
Bpin-OCL-Bpin	46.0	6.0

The obtained inflection points (Table 1) calculated from the first derivative of the isotherms for OCL-2K, B-OCL and B-OCL-B are in good accordance with those reported in literature[24] and also with those derived by X-ray data for an orthorhombic unit cell of PCL ($a = 0.748 \pm 0.002$ nm, $b = 0.498 \pm 0.002$ nm, $c = 1.726 \pm 0.003$ nm) which is $A = 37.0$ Å^2.[29-31] Bpin-OCL and Bpin-OCL-Bpin exhibit MMA_i values of 46 Å^2 and 47 Å^2 probably caused

by the larger phenylboronic acid pinacol ester end-groups leading to longer and spatial enlarged OCL chains within the Langmuir layer. Contrary, the acid end groups are more oriented to the water subphase and therefore do not contribute to *MMA* enlargement compared with OCL. At the inflection point the monolayer is dense packed and further compression of the film leads to a transition from 2D to 3D state by formation of crystallites and the growth of spherulites[32] which is visualized by BAM (Figure 2).



OCL-2K Bpin-OCL B-OCL

Figure 2. BAM images of Bpin-OCL, B-OCL, and OCL-2K layers on water above π_{max} at 22 °C.

The formation of crystallites above the maximal surface pressure π_{max} is confirmed for all investigated oligomers. Further compression of the film leads to an increase in the size and amount of the formed crystallites. Interestingly, the bulky end groups phenylboronic acid pinacol ester do not influences the OCL crystallization significantly, which indicates that the chain folding in OCL segments is still the dominant process. Phenylboronic acid end-capped OCLs form larger crystallites than the OCL-2K does at the same compression speed of 10 mm·min^{-1}. Probably, intermolecular interaction between the phenylboronic acid groups influences the crystallization growth rate.

For degradation experiments by LMD it is essential to avoid any crystallization or aggregation processes in the monolayers. To exclude those undesired processes the films were investigated at a constant surface pressure in the range below π_i (π = 6 – 8 mN·m^{-1}) over a

period of 2 h. No crystallites were formed for all samples under these experimental conditions (proofed by BAM, not shown). It is also verified that during these morphological investigation no hydrolytic degradation of PCL chains occur and the water evaporation is negligible.

3.2 Enzymatic Degradation of Langmuir Monolayers

Figure 3 shows degradation isotherms of OCL-2K, Bpin-OCL and Bpin-OCL-Bpin Langmuir monolayers at different temperatures.

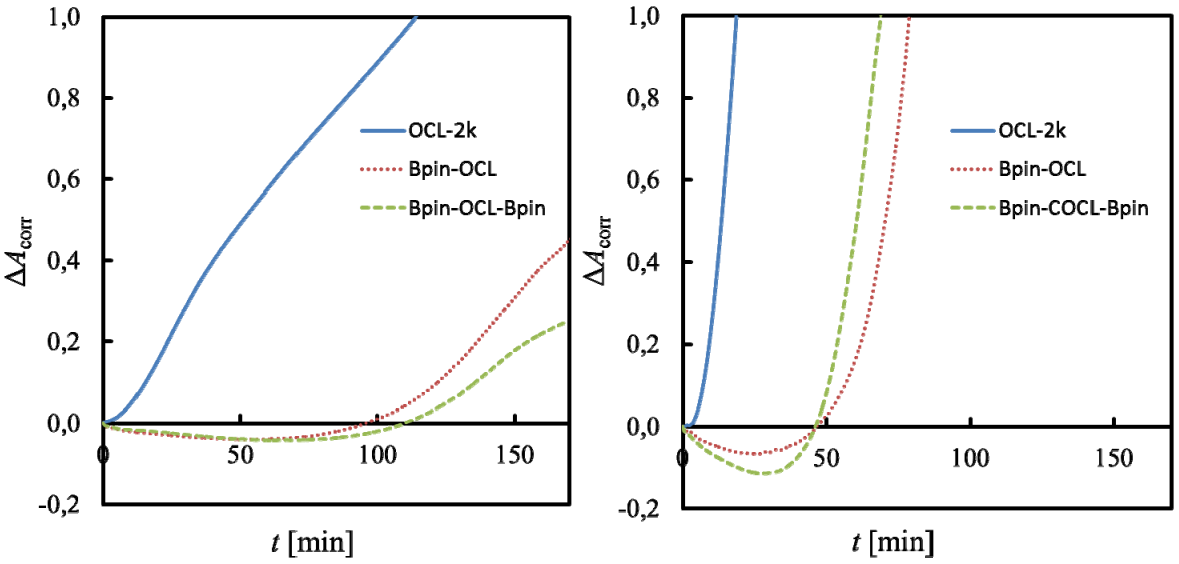


Figure 3. Enzymatic degradation isotherms of OCL-2K, Bpin-OCL and Bpin-OCL-Bpin at 22 °C (left) and 37 °C (right).

After enzyme injection into the subphase a fast degradation of OCL 2K is detected immediately. The shape of the degradation isotherm proves a random chain scission mechanism in OCL Langmuir monolayers.[4]

Contrary, for Bpin-OCL and Bpin-OCL-Bpin Langmuir monolayers significant shifts of the degradation isotherms are observed compared to pure OCL. At 37 °C an initial increase of the area (ΔA_{corr} data are negative!) occurs within the first 25 min after injection of enzyme. At

23 °C this process takes about 65 min. Only after these lag times the OCL monolayer degradation proceeds with the same slope as for OCL indicating an identical degradation behavior. The pure OCL Langmuir monolayer does not show this effect and hence, the enzyme incorporation into the Langmuir end-capped OCL layer is probably realized by interactions with the boronic acid pinacol ester groups. Also, the amount of phenylboronic acid pinacol ester end groups does not significantly change the incorporation of enzyme molecules into the monolayer and the retardation of the degradation process. Such pronounced shifts of the degradation isotherms cannot be caused by small differences in the molecular weight. It has been shown earlier that OCLs with molar masses below 10000 $g \cdot mol^{-1}$ exhibit almost identical degradation isotherms under the same experimental conditions.[33] These results suggest that the functionalization of OCL with phenylboronic acid pinacol ester does not change its Langmuir degradation behavior by any inhibition of the degradation reaction. But, the degradation process is retarded by *Pseudomonas cepacia* lipase embedding into the film.

The degradation isotherms of B-OCL and B-OCL-B at 22 °C and 37 °C exhibit the same behavior than Bpin-OCL and Bpin-OCL-Bpin showing that an enhancement of the acidity by an increase of the amount of pure boronic acid does not influence the incorporation of enzyme molecules or the retardation of the monolayer degradation (See supporting information Firgure S3).

4. Conclusion

End-capping of OCL with phenylboronic acid pinacol esters influences the compression behavior of the Langmuir monolayers due to the bulky effect of the end-groups. The layer´s compression behavior of OCL end-capped with phenylboronic acid is not affected by the end groups. Probably, the orientation of the acid to the water subphase compensate any bulky

effects. At surface pressures higher than the inflection point of the Langmuir isotherm the chain folding of OCL segments is induced and the subsequent formation of crystallites and spherulites occurs. Interestingly, the crystallization is not significantly hindered by Bpin end-groups compared to unsubstituted OCL. Contrary, the pressure induced crystallization of the B-OCLs at the air-water interface exhibit a modified rate resulting in larger spherulites which is probably caused by intermolecular interactions of the acid functions.

In contrast to OCL Langmuir monolayers phenylboronic acid and phenylboronic acid pinacol ester end-capped OCL are obviously able to incorporate enzymes of *Pseudomonas cepacia* lipase over a certain period of time and in dependence on subphase temperature. The embedding process of enzyme molecules does not inhibit the ongoing enzymatic degradation process, which follows a random chain scission mechanism.

The functionalization of a certain polymer with phenyl boronic acid groups is a novel approach to investigate their interactions with proteins at the air-water interface. A specific binding of those proteins like *Pseudomonas cepacia* lipase to the polymer monolayer could allow studying the enzymatic activity of those bound proteins and the influence on the degradation process. It also opens a way to study the impact of specified and unspecified protein interactions on the polymer monolayer morphology and on its degradation behavior.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author

Acknowledgments

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Keywords: Oligo(ε-caprolactone)s, Langmuir monolayer, phenylboronic acid, phenylboronic acid pinacolate, enzymatic degradation, Brewster angle microscopy

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