arTs Università degli Studi di Trieste
Archivio della ricerca – postprint

Functionalization of Enzymatically Synthesized Rigid Poly (itaconate)s *via* Post-Polymerization Aza-Michael Addition of Primary Amines

Alice Guarneri,[a, b] Viola Cutifani,[a] Marco Cespugli,[a] Alessandro Pellis,[a, c] Roberta Vassallo,[a] Fioretta Asaro,[a] Cynthia Ebert,[a] and Lucia Gardossi[a,*]

[a] Laboratory of Applied and Computational Biocatalysis, Dipartimento di Scienze Chimiche e Farmaceutiche, Università degli Studi di Trieste, Via Licio Giorgieri 1, 34127, Trieste, Italy
Fax: +39 040 52572
E-mail: gardossi@units.it

[b] Laboratory of Organic Chemistry, Wageningen University & Research, Stippeneng 4, 6708 WE Wageningen, The Netherlands

[c] University of York, Department of Chemistry, Green Chemistry Centre of Excellence, YO10 5DD, York, UK

Abstract: The bulky 1,4-cyclohexanedimethanol was used as co-monomer for introducing rigidity in lipase synthetized poly(itaconate)s. Poly(1,4-cyclohexanedimethanol itaconate) was synthetized on a 14 g scale at 50 °C, under solvent-free conditions and 70 mbar using only 135 Units of lipase B from *Candida antarctica* per gram of monomer. The mild conditions preserved the labile vinyl group of itaconic acid and avoided the decomposition of 1,4-cyclohexanedimethanol, both observed in chemical polycondensations. Experimental and computational data show that the enzymatic polycondensation proceeds despite the low reactivity of C_1 of itaconic acid. The rigid poly(1,4-cyclohexanedimethanol itaconate) was investigated in the context of aza-Michael addition of hexamethylenediamine and 2-phenylethylamine to the vinyl moiety. The enzymatically synthesized linear poly(1,4-butylene itaconate) was studied as a comparison. The two oligoesters (Molecular Weights ranging from 720 to 2859 g mol^{-1}) reacted on a gram scale, at 40–50 °C, at atmospheric pressure and in solvent-free conditions. The addition of primary amines led to amine-functionalized oligoesters but also to chain degradation, and the reactivity of the poly(itaconate)s was influenced by the rigidity of the polymer chain. Upon the formation of the secondary amine adduct, the linear poly(1,4-butylene itaconate) undergoes fast intramolecular cyclization and subsequent degradation via pyrrolidone formation, especially in the presence of hexamethylenediamine. On the contrary, the bulky 1,4-cyclohexanedimethanol confers rigidity to poly(1,4-cyclohexanedimethanol itaconate), which hampers the intramolecular cyclization. Also, the bulkiness of the amine and the use of solvent emerged as factors that affect the reactivity of poly(itaconate)s. Therefore, the possibility to insert discrete units of itaconic acid in oligoesters using biocatalysts under solvent-free mild conditions opens new routes for the generation of bio-based functional polymers or amine-triggered degradable materials, as a function of the rigidity of the polyester chain.

Keywords: itaconic acid; bio-based polyesters; enzymatic polycondensation; 1,4-cyclohexanedimethanol; aza-Michael addition; primary amines

Introduction

Since the last decade a wide range of structurally bio-based monomers, produced via fermentation or chemical transformation, was made available at industrial scale to mitigate the environmental impact of fossil-based plastics.[1]

In particular, itaconic acid (IA) is a diacid obtainable through fermentation of *Aspergillus terreus*[2] and with structural similarity to the fossil-based acrylic and methacrylic acids. IA offers opportunities for introducing molecular complexity because of the vinyl moiety and the 1,4 carboxylic functionalities.[3] Applications for this kind of polymers are widespread and range from dental materials,[4] elastomers,[5] drug-delivery

and other biomedical and biotechnological applications.[6] Nevertheless, there has only been little work in the field of poly(itaconate)s because the chemical polycondensation of IA takes place at temperature above 150 °C and leads to Ordelt saturation, cross- linking and isomerization of the C=C bond.[7] While the radical reactions were avoided using inhibitors such as MeHQ, BHT or quinol,[4,5] no effective solution has been identified so far to prevent the attack of the diol to the vinyl group via oxa-Michael addition (Ordelt saturation). At the best of our knowledge, the biocatalysed polycondensation is the only route for obtaining poly(itaconate)s with the vinyl groups fully preserved for further post-polymerization functionalizations, since this approach allows working under mild conditions and below 70 °C.[8]

Notably, the final objective of enzymatic polycondensation of IA is not the synthesis of long chain polyitaconates, but rather the controlled insertion of discrete units of IA within differently structured polyesters in order to develop new bio-based functional materials, which are not achievable trough conventional chemistry.[9] In particular, the *exo* double bond of IA is a suitable acceptor for aza-Michael additions,[10,11] in analogy with reactions performed with the terminal double bond of various acrylates.[12] Secondary amines are known to undergo addition to different secondary unsaturated diacids and their polymers.[10] A recent study reported the post-polymerization modification of linear poly(itaconate)s and other unsaturated polyesters using diethylamine, observing a temperature-dependent reversibility of the reaction.[10]

The aza-Addition of primary amines to IA and poly (itaconate)s appears more challenging since they lead to the fast formation of a secondary amine that promptly attacks the acyl carbon (C_4), forming stable five-member lactam rings. This reaction has been customized for the synthesis of various IA derived pyrrolidones, usable as precursors of biologically active agents[13] and bio-polyamides.[14] Therefore, because of the lactamization reaction, only secondary amines have been used for post-polymerization modifications of poly(itaconate)s.[10,15]

At the best of our knowledge, only An Lv and coworkers reported[16] the aza-Michael addition of primary amines to chemically synthesized linear poly (itaconate)s. The authors demonstrated that the intramolecular cyclization can be controlled by increasing the steric hindrance of the primary amines (e.g. *sec*-butylamine) as long as the reaction temperature is maintained below 0 °C. However, such a low temperature made necessary the use of organic solvent to dissolve the oligoesters, which were modified on mg scale. Notably, the functionalized poly(itaconate)s had to be stored at −20 °C and by increasing the temperature to 37 °C the lactamization and degradation took place promptly.[17]

In order to exploit the aza-Michael addition of primary amines to poly(itaconate)s for useful applications and under mild and practical conditions, in the present manuscript we have investigated the reactivity of two enzymatically synthesised poly(itaconate)s towards the addition of the primary aliphatic hexamethylenediamine (HMDA) and the aromatic 2-phenylethylamine (PEA). More specifically, we have analyzed the possibility to control the incidence of the lactamization and subsequent degradation of the poly (itaconate) chain by introducing the rigid diol cyclohexanedimethanol (CHDM) and by increasing the bulkiness of the amine.

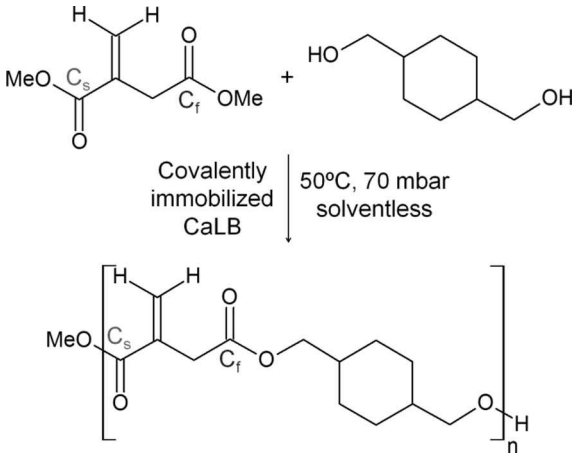
The rigid poly(1,4-cyclohexanedimethanol itaconate) and the linear poly(1,4-cyclohexanedimethanol itaconate) were synthetized by exploiting the ability of lipase B from *Candida antarctica* (CaLB) (EC 3.1.1.3) to accept different diols as substrate and to work under extremely mild conditions that preserve both the vinyl group of IA and the labile CHDM diol.

Results and Discussion

Enzymatic Synthesis of Poly (1,4-Cyclohexanedimethanol Itaconate)

CHDM is a cyclic diol currently produced at industrial level from fossil sources, although there are different routes for its synthesis starting from renewable terephthalic acid.[18] Recently CHDM attracted the interest of polymer chemists because it showed to be an effective co-monomer for enhancing the mechanical properties of 2,5-furandicarboxylic acid-based polyesters. Moreover, CHDM modified copolymers showed mechanical properties comparable to poly(ethylene terephthalate) and even better barrier properties than this polymer.[19] Even though CHDM achieved good performance in modification of poly(ethylene furanoate), the conventional polycondensation of CHDM requires temperature around 240–280 °C due to the high boiling point of CHDM. More importantly, the elevated temperature promotes decomposition and coloration of the final polyesters.[19]

In previous studies we have demonstrated that CHDM is a suitable diol for the enzymatic polycondensation of dimethyl itaconate (DMI),[8b] although the C_1-acyl group[8a] of DMI (C_s in Scheme 1) suffers from a poor reactivity caused by the conjugation with the C=C bond. Following the computational and experimental evidences acquired so far, we have enzymatically synthetized poly(1,4-cyclohexanedimethanol itaconate) on a 14 g scale without any radical quencher or solvent and at only 50 °C in order to preserve the labile vinyl group and the degradable CHDM (Scheme 1). The transesterification of C_s was boosted by enlarging



Scheme 1. Solvent-free enzymatic polycondensation of DMI with CHDM using covalently immobilized CaLB as the catalyst. C_f: fast reacting acyl group. C_s: slow reacting acyl group.

the surface available for enzyme-substrate interactions. As the porosity of the immobilization carrier is of little aid when dealing with very viscous systems, but it is rather a risk for substrate and product sequestration, the widening of the external surface of the carrier remains the only option. Conversely, the reaction was performed using only 135 U of CaLB per g of monomers but covalently immobilized on a large volume of an epoxy methacrylic carrier, used in 40% w/w on monomers basis. It must be noted that in the previous studies we had employed 240 U of CaLB per g of monomers.[8b] At the best of our knowledge, there are no further examples of synthesis of poly(1,4-cyclohexanedimethanol itaconate) (PCI) catalyzed by covalently immobilized enzymes. Most studies on biocatalyzed polycondensation report the use of 10% w/w of the commercial formulation Novozymes 435 that is prepared by physical adsorption of the enzyme, which detaches partially from the carrier.[9c,20]

The use of a covalently immobilized CaLB prevented the contamination of the product (see experimental section) by the enzyme while enabling and efficient recycling of the biocatalyst.[8,21,22] As discussed by Ansorge-Schumacher and Thum, the above mentioned factors, along with the mechanical integrity of the biocatalyst, are of primary importance for the viability of biocatalytic industrial processes, especially in the cosmetic sector.[23] Actually, despite the high viscosity of the reaction mixture, the integrity of the biocatalyst and the efficiency of the mass transfer were assured by the application of the thin-film technology, which was previously described.[8a,24]

In the present study the reaction led to an array of oligomers made by 6–9 units, as also confirmed by GPC analyses ($M_n = 550$, $M_w = 720\ g\,mol^{-1}$; $Đ = 1.32$).

The CHDM used in the polycondensation was a mixture of 60% of the *trans* and 40% of the *cis* isomers as demonstrated by ^{1}H-NMR spectra associated with DQ-COSY analysis and HSQC analyses, which are reported in Figures S1 and S2 of Supporting Information (SI). The products of the transesterification were characterized by GPC, ESI-MS, ^{1}H-NMR and DQ-COSY analyses (see SI, Figures S3–6), after recovery in dichloromethane (DCM) and filtration of the biocatalyst, without further purifications. NMR analysis confirms the preservation of the vinyl moieties and allowed the quantitative evaluation of the formation of the different isomers as well as the extent of the esterification of the fast and slow reactive acyl groups of DMI (see ^{1}H-NMR spectra in SI, Figure S6).

The different reactivity of the *cis* and *trans* isomers of CHDM in the enzymatic polycondensation was evaluated by calculating the ratio between the new signals of the esterified $–CH_2OH$ methylenic protons and the total of the signals of all $–CH_2OH$ methylenic proton signals. Conversely, 65% of hydroxyl groups resulted esterified (Table 1). A detailed analysis of the esterification of the *cis* and *trans* stereoisomers was carried out with the aid of a HSQC and DQ-COSY spectra (See SI, Figure S4).

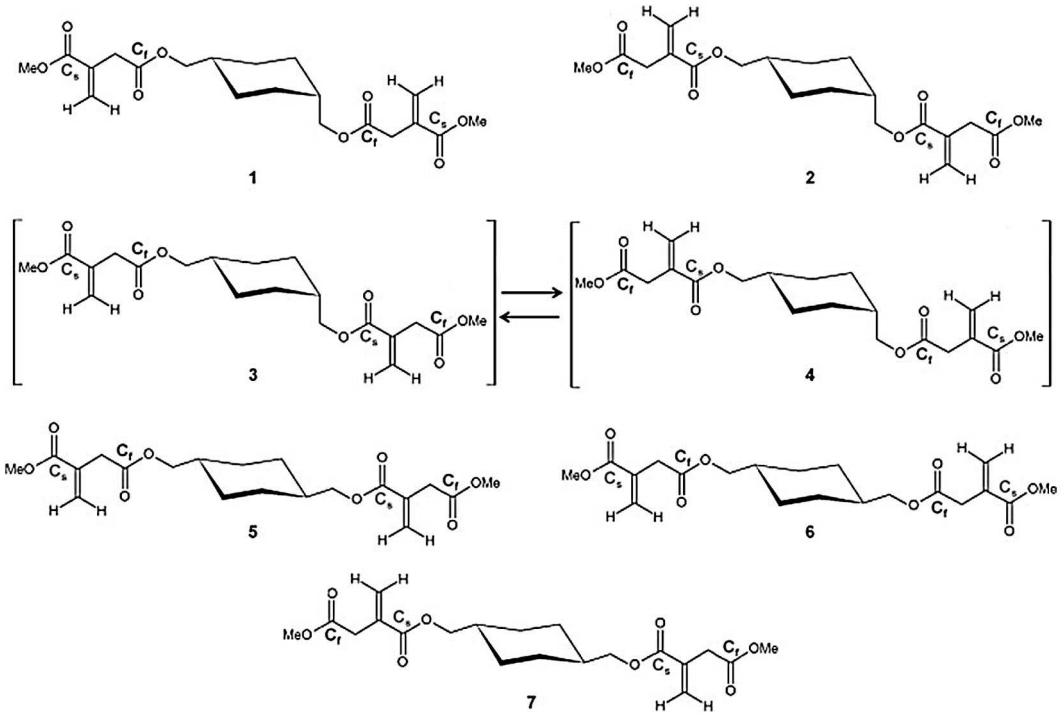
When the relative presence of the two different stereoisomers is considered (40% *cis* and 60% *trans*) the two isomers appear to react at a comparable extent but the *trans* isomer shows a more evident preference for the C_f (Table 1).

Table 1. Reactivity of the stereoisomers of CHDM with respect to the two acyl groups of DMI. The values reported in brackets were normalized against the relative concentration of the cis (40%) and trans (60%) stereoisomers.

	Cis (%)	*Trans* (%)	Total (%)
C_f	12 (29)	30 (50)	42
C_s	8 (20)	15 (25)	23
React total	20 (49)	45 (75)	65

ESI-MS shows that the trimer ABA (where A is the itaconate monomer and B is the diol) does not accumulate and this is the main pre-requisite for the elongation of the polyester chain. In fact, when the diol is acylated by two DMI units through two C_f, the resulting trimer has only two slow reacting acyl groups available for the further elongation and, therefore, the polycondensation stops.[8b]

The stereoisomerism of CHDM offers an opportunity for introducing structural complexity in the polyester chain. Therefore we used computational methods to investigate the ability of CaLB to catalyze the elongation of the different isomers of the ABA trimer, which are reported in Scheme 2.



Scheme 2. The isomers and conformers of the ABA trimer obtainable through the enzymatic polycondensation of CHDM with DMI using CaLB as the biocatalyst.

When both hydroxy groups of CHDM undergo acylation, the *trans* and *cis* configurations potentially lead to the formation of three isomers each. Two products have both OH groups esterified with either two C_f (**1**, **6**) or with two C_s (**2**, **7**), the third one presents one C_f and one C_s as acyl groups. The *trans* configuration allows, in all cases, for the placing of both substituents in equatorial positions (**5**, **6**). The *cis* configuration has one bond in equatorial and the other in axial position (**3**, **4**). Therefore, the *cis* isomer acylated by one C_f and one C_s has two possible conformers.

The computational analysis was carried out by docking the different isomers and probable conformations of ABA, and then by evaluating the productive poses (Near Attack Conformations, Figure 1).[25]

Table 2 reports the productive poses causing the elongation (E) of the polymer through the formation of the acyl-enzyme on the terminal acyl groups. They can be quantitatively compared with poses that refer to the attack of the internal acyl groups already bound to CHDM. In the latter case it is assumed that the reaction leads to the degradation (D) of the polyester.

Notably, the *cis* product **1**, obtainable from the *cis* CHDM acylated by two C_f, shows the lowest probability of elongation (elongation/degradation = 0.25), whereas the *cis* trimer **2** (CHDM acylated by two C_s) has the highest chances of elongation (elongation/degradation = 4.33). Data also indicate that the *trans* product **5** (CHDM acylated by one C_f and one C_s) presents the highest chances of elongation (elongation/degradation = 3.00) and predominantly through the

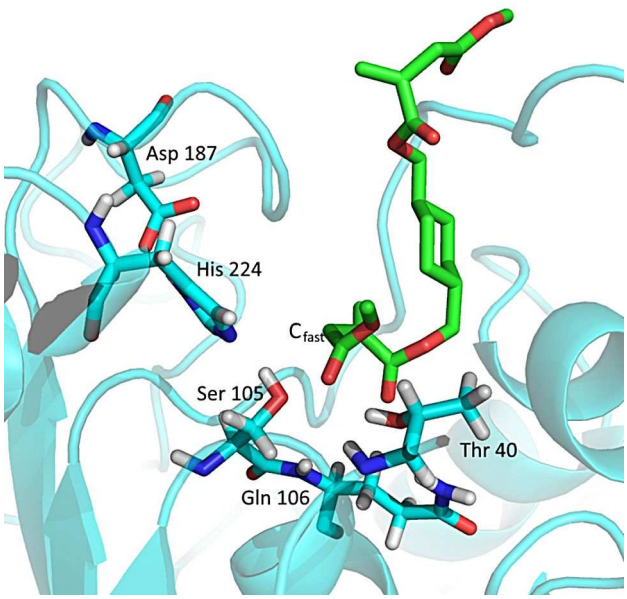


Figure 1. Docking of the *trans* trimer ABA **5**, having the CHDM acylated by one C_f and one C_s. The pose illustrates the Near Attack Conformation for the formation of the acyl-enzyme between the catalytic Ser 105 and the fast-reacting external acyl group.

Table 2. Productive poses for the attack of C_s and C_f computed by simulating the Near Attack Conformations of the different isomers 1–7 of ABA trimer docked in the CaLB active site. The data distinguish between the attack of the catalytic Ser to a terminal acyl group of DMI (E = Elongation) and an intra-chain ester group linking DMI and CHDM (D = Degradation).

ABA isomer	E C_s	D C_f	E C_f	D C_s	E/D
1	2	8	–	–	0.25
2	–	–	13	3	4.33
3	4	3	5	2	1.80
4	4	3	5	4	1.29
5	1	3	11	1	3.00
6	5	7	–	–	0.71
7	–	–	14	5	2.80

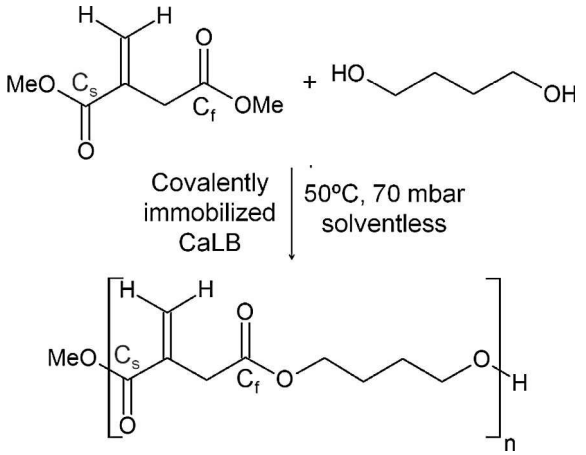
highly reactive C_f terminal (11 productive poses against 1 for C_s). Therefore, the poor reactivity of the C_s is also accompanied by lower chances to be attacked by the enzyme (31 productive poses of for C_s acylation vs 72 productive poses for C_f acylation), probably because of the steric hindrance caused by the methylene group. Overall, the docking analysis indicates that it is crucial to boost the reactivity of the C_s already at the level of the DMI monomer, because the slow-reactive conjugated acyl group is even more hampered at the stage of the trimer ABA. Therefore, the computational analysis combined with the experimental data confirm that the experimental conditions applied to the polycondensation promoted the transesterification of C_s at the level of the DMI monomer, thus leading to chain elongation.

Finally, while in the first step of the CHDM acylation there is no stereo-preference between the *cis* and *trans* isomers, the docking analysis indicates that the elongation is more probable at the level of the *trans* intermediate ABA **5**.

Enzymatic Synthesis of Linear Poly(1,4-Butylene Itaconate)

To investigate the effect of polymer rigidity on the aza-Michael addition, the linear poly(1,4-butylene itaconate) (PBI) was synthesised for comparison, using 1,4-butanediol (BDO) as co-monomer (Scheme 3).

Our previous experimental and docking studies indicate that the elongation of poly(itaconate)s occurs with more difficulty when BDO is used as co-monomer.[8b] Therefore, 50% w/w of three different low-loaded formulations of covalently immobilized CaLB were tested (see experimental) on a scale ranging from 2 to 16 g. Besides using two preparations immobilized on epoxy methacrylic carriers (specific activities of 526 and 338 U/g_{dry} respectively) we also employed activated rice husk, a renewable and inexpensive support (specific activity of the formulation = 309 U/g_{dry}).[21,26,27]



Scheme 3. Solvent-free enzymatic polycondensation of DMI with BDO using covalently immobilized CaLB as the catalyst.

The efficiency of the polycondensation reactions was confirmed by GPC, ^{1}H-NMR and ESI-MS spectra (see SI, Figures S7–10 and S17–20). The longest oligomers were obtained using the rice husk carrier ($M_n = 1600$ g mol^{-1} and $M_w = 2900$ g mol^{-1}; Đ = 1.76), thus indicating the possibility to mitigate the negative environmental and economic impact deriving from the use of large amounts of fossil based carriers.[26] The best formulation of CaLB immobilized on methacrylic resins gave products with $M_n = 1100$ g mol^{-1} and Mw = 1800 g mol^{-1} (Đ = 1.61). As previously reported,[27] the low density (0.4 g mL^{-1}) of rice husk allows the distribution of the enzyme on a wider surface of carrier as compared to the methacrylic resin (density ~1.1 g mL^{-1}). Hence, the volume of the rice husk carrier was at least double on a weight basis as compared to methacrylic resins and that improves the accessibility of the enzyme.

The ^{1}H-NMR signals of vinyl protons (5.73–6.33 ppm) confirmed that the C=C was fully preserved throughout the reactions.

The implementation of efficient protocols for the enzymatic synthesis of linear and cyclic poly(itaconate)s prompted us to investigate the post-polymerization modification of the vinyl pendants. However, to better understand the reactivity of the C=C bond of itaconic acid, a model reaction between the DMI monomer and a primary diamine was studied first.

Aza-Michael Addition of Hexamethylenediamine (HMDA) on the DMI Monomer

The linear hexamethylenediamine (HMDA) was used as nucleophilic donor for the aza-Michael addition to the monomer DMI. The reaction was carried out simply by simply mixing DMI with 0.2 equivalents of

HMDA at 50 °C without any addition of solvents and under atmospheric pressure for 20 h. The mild heating allows the melting of both reagents while avoiding the heat-driven isomerization or crosslinking processes involving the double bond moiety.

The use of a linear primary di-amine was motivated by the interest of creating crosslinks through the amine functionalities or to introduce free primary amine groups. In principle, the latter could be exploited either for increasing the polarity of the products or for the anchoring of biomolecules.

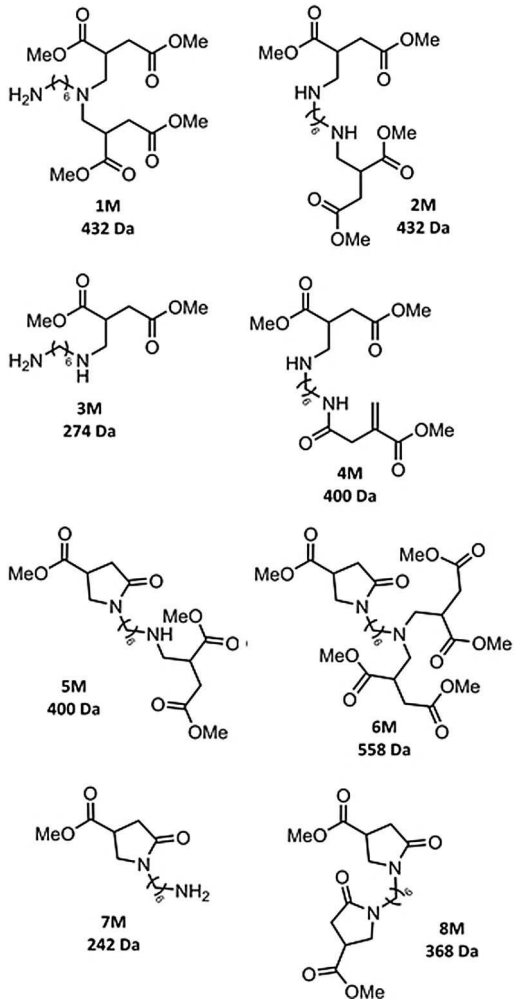
Upon the addition of 0.2 equivalents of HMDA, the aza-Michael reaction proceeded rapidly despite the absence of any catalyst. After only 3 min (time required for the complete melting of the solid reagents into an homogeneous viscous system) the ^{1}H-NMR spectrum showed the disappearance of 47% of vinyl groups. Besides the occurrence of the amination of the double bond the spectra also indicated the subsequent lactamization and the formation of differently cross-linked adducts and pirrolydones. At 20 h 54% of the vinyl groups reacted but the aza-Michael mono- and bis-adducts represent less than 2% of the products since 38% of the reacted alkenes led to subsequent lactamization. The fast lactamization process competes with the Michael addition of the newly-formed secondary amine to another double bond. The percentage of reacted C=C is above the maximum theoretical value of 40% (0.2 equivalents HMDA were used in respect to the vinyl moiety of DMI) and this indicates the formation of bis-adducts and crosslinked species, also confirmed by the registered molecular weights (see SI, Figures S13–16 and Table S2).

After 20 h there is also evidence that about 10% of DMI underwent isomerization. The thermal rearrangement of DMI generally occurs at higher temperatures (> 150 °C),[7c] but the basic HMDA ($pK_{a1} = 10.762$; $pK_{a2} = 11.857$) catalyzes the isomerization of unsaturated esters.[28] Dimethyl citraconate and mesaconate are less reactive as Michael acceptors in respect to the itaconate and they did not react with the diamine,[12b,29] as demonstrated by the persistence of mesaconic esters after 20 h of exposure to HMDA.

No signals of linear amides were detected in the ^{1}H-NMR spectra (Figure 2) even after 20 h of reaction. The negligible competition of aminolysis of the methyl esters was confirmed by a control reaction between HMDA (0.2 equivalents) and dimethyl adipate (DMA), which presents two equally reactive acyl groups. In the case of DMA, only 6% amino groups were acylated after 20 h (see SI, Figures S11–12 and Table S1).

The mass spectra of the product after 90 min, 3 h and 20 h show the same chemical species, including pyrrolidones, acyclic mono- and bis-adducts, as well as cross-linked dimers and trimers (Scheme 4).

As the concentration of the primary diamine HMDA decreases, the lactamization slows down and the secondary amine attacks the C=C of the DMI in excess.



Scheme 4. Structures of potential products detected by ESI-MS after 20 h of reaction between DMI and HMDA (0.2 eq.).

Aza-Michael Addition of Primary Amines to PCI and PBI

This complex scenario of reactivity observed in the reaction between the monomer DMI and the linear HMDA, suggested to investigate the post-polymerization aza-Michael addition of primary amine to PCI and PBI by varying the concentration of the nucleophile as well as its bulkiness.

HMDA and the aromatic monoamine, 2-phenylethylamine (PEA), were added stepwise to enzymatically synthesized PCI and PBI (see SI, Figures S21–76 for product characterizations), with aliquots of 0.2 equivalents, calculated based on itaconic C=C bonds present in the reaction mixture (Scheme 5). The aza-Michael additions of HMDA and PEA to the oligoesters were carried out at 40 and 50 °C respec-

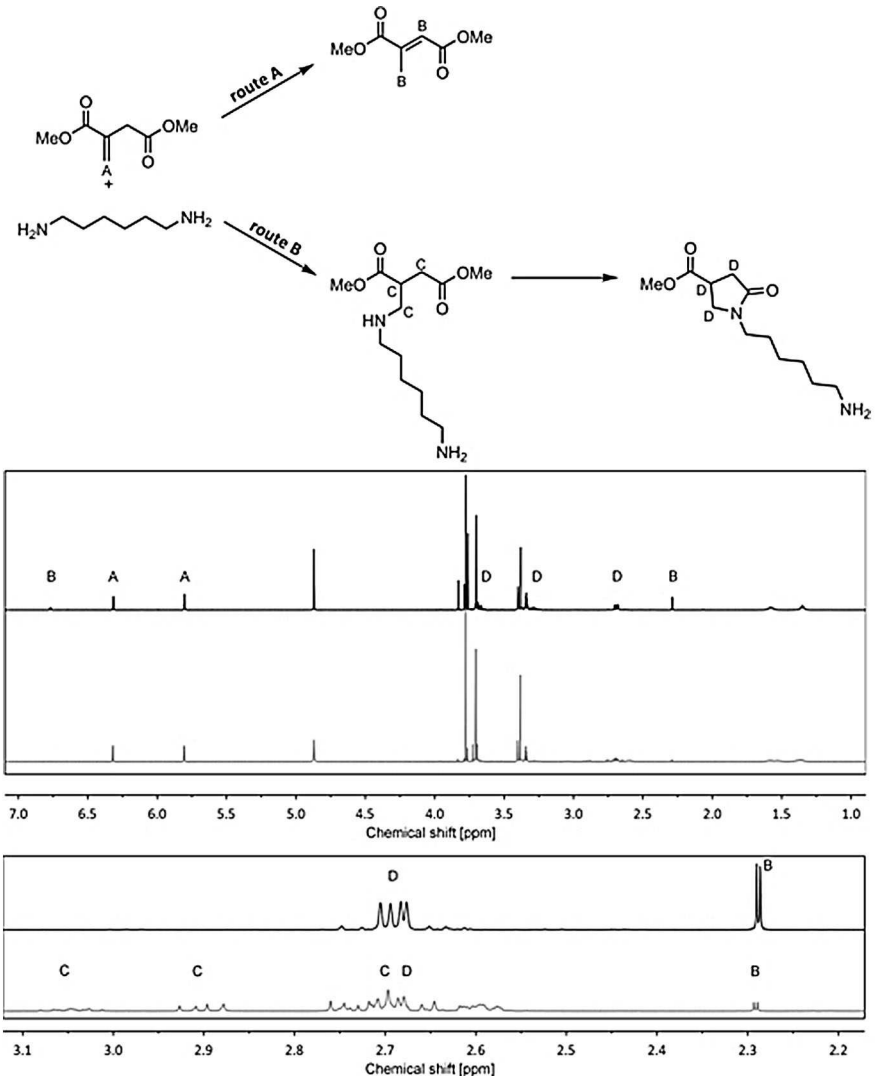
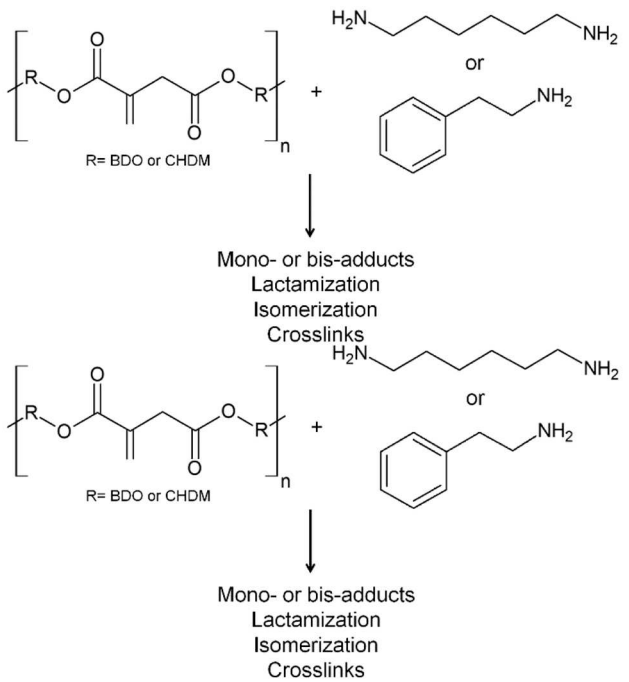


Figure 2. Top: main reactivity of the vinyl group of DMI in the presence of HMDA (0.2 eq.), Route A: base catalyzed isomerization; route B: aza-Michael addition and subsequent lactamization. Bottom: ^{1}H-NMR spectra in CD_3OD of the crude reaction mixture at 3 min (grey trace) and 20 h (black trace) and zoom of the spectra from 3.1 to 2.2 ppm.

tively. As in the previous case, the aza-Michael additions were carried out at 50 °C, under atmospheric pressure and without solvent. The reaction between HMDA and PCI was reproduced also in DCM to verify the effect of the organic solvent. At the end of this procedure the amine groups added to the oligomers were in excess (1.2 equivalents) in the case of the HMDA, whereas for the monoamine there was a maximum of 0.6 equivalents of amine present in the reaction mixture.

The attack of the C=C bond and the subsequent formation of the products were monitored by means of bi-dimensional DQ-COSY and HSQC (see SI, Figures S22–26). The disappearance of the $-CH_2$ proton signals provides a quantitative evaluation of the saturation of the vinyl moiety and Figure 3 describes the patterns observed with PBI and PCI by varying the concentration of the two amines. The di-amine leads, as expected, to a faster decrease of the vinyl signals. Some adducts and even lactams are observable since the very beginning of the reaction.

At t = 456 min, the reaction between PBI and 0.6 eq. of HMDA (corresponding to 1.2 equivalents of amine groups) leads to the complete saturation of all C=C bonds (see SI Figures 22–25). Notably, the addition is much slower in the case of PCI and HMDA (see SI Figures 60–66), where the conversion reaches only 82%, most probably because of the rigidity conferred by the cyclic CHDM. A further cause might reside in the higher viscosity of the mixture. However, when DCM was used as solvent to reduce the viscosity (1.16 g of PCI in 20 mL of DCM), the rate of the same reaction slowed down significantly and the conversion was only 54% after 20 h (see SI, Figures 73–76).



Scheme 5. Schematic representation of the post-polymerization modification of PCI and PBI *via* aza- Michael addition of HMDA and PEA.

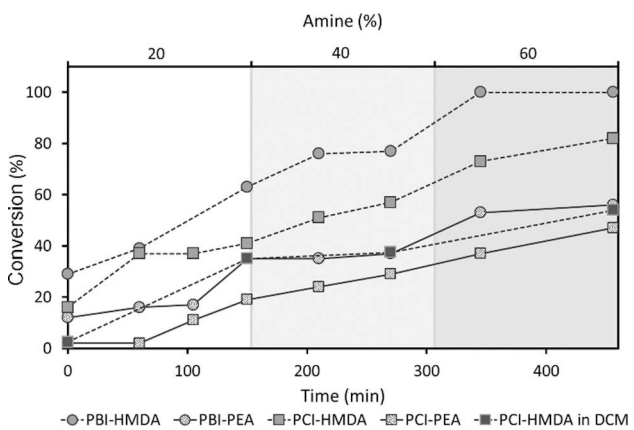


Figure 3. Reaction of C=C bond of PCI and PBI upon addition of PEA and HMDA, expressed as a function of the disappearance of vinyl ^{1}H-NMR signals over time. PCI: squares, PBI: circles. PEA: pattern fill and solid conjunction line, HMDA: grey fill and dashed conjunction line. Additions were made at time 0, 105 min and 285 min, as represented by a gradient of grey coloured block.

The reaction of the linear PBI with 0.6 eq. of the aromatic amine PEA (see SI, Figures 36 and 38 and 52–58) led to almost a quantitative addition of the monoamine (maximum theoretical yield = 60%). PEA reacts more slowly with the cyclic PCI oligoester and leads to 47% of conversion after 456 min (see SI, Figures 43 and 45 and 66–72). Overall, the rigidity of

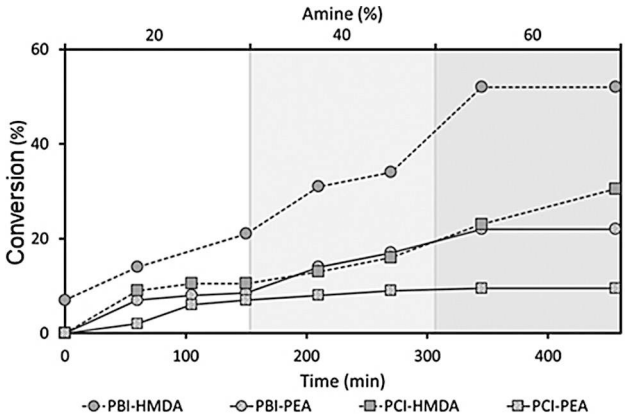


Figure 4. Polymer degradation caused by the addition of PEA or HMDA to PCI and PBI. PCI: squares, PBI: circles. PEA: pattern fill and solid conjunction line, HMDA: grey fill and dashed conjunction line. Additions were made at time 0, 105 min and 285 min, as represented by a gradient of grey coloured blocks. Additions were made at time 0, 105 min and 285 min, as represented by a gradient of grey coloured blocks.

the PCI chain, the bulkiness of the amine and the use of organic solvent appear factors that can be exploited for tuning the rate of the aza-Michael reaction.

Effect of Chain Rigidity on the Lactamization of DMI and Polyester Degradation

The rigidity of the cyclic diol CHDM resulted to affect significantly the degradation of the polyester chain by slowing down the intramolecular attack of the newly formed secondary amine to the adjacent fast-reacting acyl group (Figure 4). This reaction leads to a pyrrolidone ring and the concomitant degradation of the ester bonds of the chain, unless the C_f of DMI represents the terminal of the chain, so that only methanol is released in such case. The degradation of the polyester was monitored by evaluating the disappearance of the NMR signals of the methylenic protons of the esterified BDO and CHDM with the concomitant appearance of the signals of the free diol.

From Figure 4 it is evident that the linear oligomer PBI undergoes much faster degradation than the cyclic PCI using either the diamine HMDA or the aromatic monoamine PEA. The rate of degradation of PBI in the presence of PEA is comparable to that observed with PCI with HMDA, namely in the presence of a double concentration of amine groups.

In the case of the linear PBI, the starting oligomer mixture had 62% of –OH groups of BDO esterified and the reaction with HMDA led to the degradation of 52% of these ester bonds in 456 min of reaction (Figure 4). This is confirmed by HPLC analysis showing the degradation of the oligomers (see SI, Figures S27–33 and Table S3). IR analyses at different

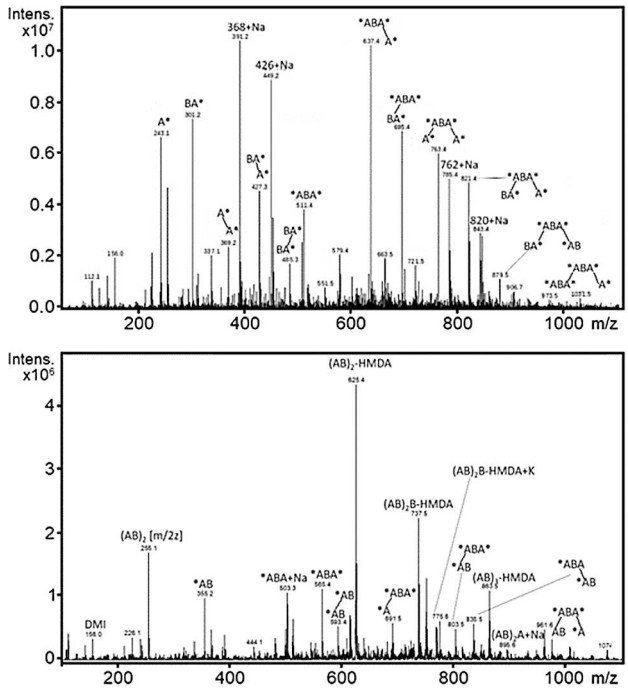


Figure 5. ESI-MS spectra (TM = 1000 Da) of the aza-Addition of HMDA to PBI (top) and PCI (bottom) at 456 min and 50 °C. A: DMI; B diol; * stays for a lactam ring; the straight-line connections indicate a crosslink.

reaction times indeed confirm the ongoing saturation of the vinylic bond and the formation of cyclic pyrrolidones. Simultaneously, the ester bond signal decreases while the free OH signal increases, proving that HMDA causes a fast degradation of the linear PBI oligomer chains (see SI, Figure S34). Notably, NMR spectra confirm the presence of the mono-adduct until 4.5 h, whereas afterwards only lactamized DMI derivatives are observed. The analysis of the reaction mixture after 456 min by means of ESI-MS (Figure 5) shows that the Michael addition of HMDA to the linear PBI yields a complex mixture of substituted pyrrolidones, also cross-linked through the diaminic bridge. Apparently, no itaconate unit was preserved from the lactamization and there is no evidence of the formation of bis-adducts, namely tertiary amines formed from two sequential aza-additions of the same N atom to two C=C bonds. The crosslinked product with highest M_w is A*BA*-A*BA*-A* (m/z = 1031.5), where A* stays for the pyrrolidone ring obtained through lactamization.

In the case of PCI, the starting mixture of oligomers had 65% of methylenic protons of CHDM esterified, which underwent a 30% decrease upon addition of 0.6 eq. of HMDA and 20 h of reaction (Figure 4). The ESI-MS analysis (Figure 5) reveals the formation of a number of crosslinked pyrrolidone derivatives, with BA*-A*BA* having the highest M_w (m/z = 803.5). Moreover, the products include an array of mono-adducts of HMDA on PCI, which bear a free amine side chain (Figure 5), as in the cases of $(AB)_2$B-HMDA (m/z = 737.5) and $(AB)_3$-HMDA (m/z = 863.5). They are visible in the spectra until 456 min, indicating their stability towards the lactamization even at 40 °C. Notably, in these stable mono adducts the cyclic diol represent the terminal of the chain, indicating that the lactamization occurs prevalently al the level of terminal DMI, which have either the C_f or the C_s esterified with methanol. In the first case the lactamization will be particularly fast because the methanol acts as a leaving group. Other products are represented by hybrid species obtained from the cross-linking between pyrrolidone derivatives and itaconate units, as in the case of BA-A*BA*-A* (m/z = 961.6).

Therefore, the cyclic CHDM is indeed effective in conferring rigidity to the ester bond thus hampering the intramolecular attack of the secondary amine to get the cycle. It is interesting to note that not cross-linked monoadducts bear a free $-NH_2$ group availablefor further functionalization of the oligomers (Figure 4).

In conclusion, there is evidence that the degradation of poly(itaconate)s triggered by primary amines can be tuned by the chain rigidity and that products of aza-Michael additions on poly(itaconate)s can be observed after 20 h at 40 or 50 °C for 20 h without specific control of pressure or use of inert gases. Although the non-optimized reaction conditions led, in this case, to a complex mixture of products, data indicate that there is room for further optimization of reaction parameters for achieving the control of the reaction, while avoiding extreme cooling[16] and making this post-polymerization functionalization of practical utility.

Effect of the Structure and Concentration of the Amine on the Lactamization

The aza-Michael reactions performed with PEA did not lead to the complete saturation of the vinyl groups because only 0.6 eq. of the monoamine were added.

The ESI-MS spectra of the PBI reacted with the aromatic and bulky PEA (see SI, Figure S35) show some oligomers that did not undergo lactamization. This appears as the main difference in respect to the reaction between PBI and the linear HMDA.

Therefore, also the bulkiness of the amine influences the lactamization. This is visible in the reaction between the cyclic PCI and the aromatic PEA, where the degradation of the polyester chain (Figure 4) proceeds very slowly, even after the third addition of the amine, as also confirmed by the GPC analysis. Interestingly, in the addition of PEA to the cyclic PCI, the polymer degradation seems to be independent from the concentration of PEA and only 9.5% of the ester bonds underwent degradation upon the addition of 0.6 eq. of amine. The ESI-MS (see SI Figure S35)

indicates that the mono-adducts are the predominant products and they are still present at long reaction times, which appears quite remarkable considering that all reactions were carried out at 50 °C. Indeed, the product $(AB)_4$-PEA (m/z = 1107.6) is still present after 456 min.

Also, in the case of addition of PEA the incidence of the lactamization is higher on the itaconate units located at the extremities of the chain. The ESI-MS and NMR spectra exclude the formation of bis-adducts, probably because of the bulkiness of the substrates.

Concerning the isomerization of the C=C bond, NMR data show that it is prevalently influenced by the amine concentration, since there is no major difference between the behaviour of PBI and PCI (see SI, Figure S77). The solvent (DCM) is one further factor preventing the isomerization, although the aza-Michael reaction carried out in organic solvent with HMDA is globally much slower, thus suggesting an effect due to the decreased basic reactivity of the diamine. The incidence of isomerization in PCI and PBI is below 8% in the presence of HMDA, which is comparable to what observed in the case of the DMI monomer (10% of isomerization after 20 h).

Conclusions

The feasibility of enzymatic polycondensation of dimethyl itaconate with either linear 1,4-butanediol or the rigid and bulky cyclohexanedimethanol sets the basis for the exploitation of the bio-based unsaturated itaconic acid monomer as a chemical platform for the design of new functionalyzed polyesters with controlled architectures and reactivities.

The present study sheds light on the structural and experimental factors that must be controlled in order to exploit the aza-Michael addition of primary amines for obtaining poly(itaconate)s with functionalized side chains or, rather, for degrading the polyesters. Although it had been widely reported that the Michael adducts (secondary amines) lead to fast lactamization and degradation of poly(itaconate)s, our results indicate that the rigidity of the co-monomer 1,4-cyclohexanedimethanol decreases the incidence of lactamization and the consequent degradation of PCI when reacted with hexamethylenediamine. Under non-optimized conditions the reaction yields to different products. Among them, mono-adducts bearing a free amine exploitable for further functionalization proved to be stable at 40 °C for at least 7 h.

The minimum incidence of lactamization was observed when the effect of the chain rigidity was combined with the bulkiness of the aromatic 2-phenylethylamine. The crucial role of chain rigidity was also confirmed by the faster degradation of the linear poly (1,4-butylene itaconate) (PBI) in the presence of both primary amines.

All aza-Michael additions were carried out on multi-gram scale at 40 or 50 °C, in the absence of any solvent or catalyst and without specific control of pressure or use of inert gases.

Notably, the synthesis of both polyesters used in this study was made possible by the optimization of their enzymatic polycondensation, taking into account both environmental and economic aspects. By working in solvent-free conditions at 50 °C and 70 mbar, not only the labile vinyl group of dimethyl itaconate was preserved, but also the degradation of the 1,4-cyclohexanedimethanol was avoided, thus obtaining poly (itaconate)s with M_w, ranging from 720 to 2859 $g\,mol^{-1}$.

The possibility to insert discrete units of itaconic acid in polyesters via enzymatic polycondensation opens new routes for the development of structurally complex bio-based functionalized materials not achievable through conventional chemistry[30] but also to materials degradable via amine-triggered reactions[31] potentially applicable in biomedical and packaging sectors.

Experimental Section

General Information

Rice husk samples were kindly donated by Riseria Cusaro (Binasco, Italy). *Candida antarctica* lipase B in the form of Lipozyme® CaLB L was purchased from Novozymes (Bagsværd, Denmark). All other reagents and solvents were of analytical grade, were purchased from Sigma-Aldrich and were used as received if not otherwise specified.

The two amines used for aza-Michael addition reactions were dehydrated at 80 °C (flash point of phenylethylamine = 90 °C) using molecular sieves, previously activated at 200 °C overnight.

NMR Spectroscopy

NMR spectra were recorded on an Oxford JEOL (270 MHz) or a Varian (400 MHz) spectrometers.

^{1}H-NMR Spectra Related to Polycondensation of DMA and BDO

Spectra were recorded on an JEOL Ex-270 spectrometer operating (270 MHz). The solvent was $CDCl_3$ if not otherwise specified. Original spectra are reported in SI.

Thin Layer Chromatography (TLC)

For the TLC analysis on silica gel, glass plates of dimensions 20 × 20 cm (Macherey-Nagel) were used. The spots were visualized by treating the silica plates with a solution mixture of $KMnO_4$/KOH (1.25/0.5%) or ninhydrin solution. The components were separated employing ethyl acetate or mixtures of ethyl acetate and n-heptane or MeOH as mobile phase.

Electrospray Ionization Mass Spectrometry (ESI-MS)

The crude reaction mixtures were analyzed on Esquire 4000 ESI-MS ion trap Bruker (Karlsruhe, Germany) instrument electrospray positive ionization by generating the ions in an acidic environment. Around 10 mg of sample was dissolved in 1 mL methanol containing 0.1% vv^{-1} formic acid. The generated ions were positively charged with mz^{-1} ratio falls in the range of 100–1000. The subsequent process of deconvolution allows the reconstruction of the mass peaks of the chemical species derived from the analysis of the peaks generated.

Gel Permeation Chromatography (GPC)

Polymeric samples were dissolved in the mobile phase (HPLC-grade THF with 250 ppm of BHT as peroxides inhibitor) to a concentration of ~2 mg mL−1. Samples were then filtered through 0.45 µm PTFE filters into glass HPLC vials. GPC was carried out at a T of 40 °C on an Agilent Technologies HPLC System (Agilent Technologies 1260 Infinity) connected to a 17369 6.0 mm ID × 40 mm L HHR-H, 5 µm Guard column and a 18055 7.8 mm ID × 300 mm L GMHHR-N, 5 µm TSKgel liquid chromatography column (Tosoh Bioscience, Tessenderlo, Belgium). The mobile phase flow rate was set to 1 mL min-1. An Agilent Technologies G1362A refractive index detector was employed for detection. The molecular weights of the obtained polymers were calculated using polystyrene calibration standards (250–70,000 g mol^{-1}) purchased from Sigma-Aldrich.

Thin-Film Reaction Systems for Enzymatic Solventless Polycondensation

The reactions were operated with a Rotavapor R-114 (BÜCHI) connected to a vacuum pump Vac® V-513 (BÜCHI) and a pressure controller V-800 (BÜCHI). Temperature was controlled by means of a Waterbath B-480 (BÜCHI).

HPLC Analyses

Inverse-phase HPLC analyses system is composed by a Syringe pump Gilson 402 connected to a Gilson 232XL Sampling injector, a Gilson 321 pump for eluents and a Column oven CTO-10AS Shimadzu. The used pre-column is Phenomenex Menex IB-Sil C8 5 µm, 125 Å, 30 × 4.60 mm connected to the column Phenomenex Gemini-NX C18 5 µm, 110 Å, 250 × 4.60 mm. Diode array detector Agilent 1100 Series was used for detection.

Lipase Hydrolytic Activity Assay for Determining Tributyrin Units (TBU)

The activity of enzymatic preparations was assayed by monitoring the tributyrin hydrolysis according to a method previously reported.[27]

CaLB immobilized Covalently on Epoxy-Methacrylic Resins EC-EP

Two formulations of immobilized CaLB were prepared using the epoxy acrylic resin Relizyme® EC-EP (average pore diameter 40–60 nm, Resindion, Milano, Italy) according to protocols previously reported.[27] The loadings of the enzyme was of 15000 and 10000 U per gram of resin. The hydrolytic activities were 526 and 338 U/g_{dry} respectively. They were determined via tributyrin lipase activity assay. More than 98% of protein was immobilized and it was verified that there was no enzyme leaching from the support using a method previously reported.[27]

CaLB Immobilized Covalently on Functionalized Rice Husk (RH-CaLB)

10.000 units of CaLB (tributyrin Units) were loaded on 1 g of dry, oxidised rice husk and functionalized with HMDA according to the previously reported protocol.[41] 72% of the protein was loaded on the carrier after 48 h of reaction at 25 °C. The final activity of the immobilized CaLB was of 316 U $g_{dry}{}^{-1}$ (tributyrin assay). At the end of the activity assay, it was verified that there was no enzyme leaching from the support.[27] The recyclability of CaLB covalently immobilized on rice husk has been demonstrated in a previous study[21] and it is the consequence of a remarkable stiffness of this natural composite material because of the high silica and the lignin content.[21]

Enzymatic Synthesis of Poly(1,4-Butylene Itaconate) Catalyzed by CaLB Covalently Immobilized on Epoxy Methacrylic Resins (263 U per g of Monomers)

The reaction was carried out using equimolar amounts of dimethyl itaconate DMI (16 mmol, 2.4950 g) and 1,4-butanediol (BDO, 16 mmol, 1.462 g) and 50% w/w of immobilized CaLB on the carrier Relizyme® EC-EP (263 U/g monomers). The thin-film enzymatic polycondensation[15] was carried in a 100-mL round-bottom flask connected to a rotary evaporator at 50 °C and 70 mbar. The requested amount of diol was divided in two aliquots, one added at the start of the reaction, the other after 6 hours. After 72 h, the reaction mixture was recovered with DCM and products were characterized after filtration of the biocatalyst and evaporation of DCM, without any further treatment. The final product appears as a transparent viscous liquid ((recovered product: 2.2 g; 56% yield).

Enzymatic Synthesis of Poly(1,4-Butylene Itaconate) Catalyzed by CaLB Covalently Immobilized on Rice Husk (158 U per Gram of Monomers)

The reaction was carried out using equimolar amounts of DMI (8 mmol, 1.2485 g) and BDO (8 mmol, 0.72 g) and 50% w/w of immobilized CaLB on the rice husk carrier (158 U/g monomers). The diol was previously treated with molecular sieves for moisture elimination. The thin-film enzymatic polycondensation[8a] was carried in a 25-mL round-bottom flask connected to a rotary evaporator at 50 °C and 70 mbar. The requested amount of diol was divided in two aliquots, one

added at the start of the reaction, the other after 6 hours. After 72 h, the reaction mixture was recovered with DCM and products were characterized after filtration of the biocatalyst and evaporation of DCM, without any further treatment. The final product appears as a transparent viscous liquid (recovered product: 1,28 g; 65% yield).

Enzymatic Synthesis of Poly(1,4-Butylene Itaconate) Catalyzed by CaLB Covalently Immobilized on Epoxy Methacrylic Resins (169 U per g of Monomers)

The reaction was carried out using equimolar amounts of DMI (0.06323 mol, 10 g) and BDO (0.06323 mol, 5.6984 g) and 50% w/w of immobilized CaLB on the carrier Relizyme® EC-EP (169 U/g monomers). The diol was previously treated with molecular sieves for moisture elimination. The thin-film enzymatic polycondensation[15] was carried in a 250-mL round-bottom flask connected to a rotary evaporator at 50 °C and 70 mbar. The requested amount of diol was divided in two aliquots, one added at the start of the reaction, the other after 6 h. After 72 h, the reaction mixture was recovered with DCM and products were characterized after filtration of the biocatalyst and evaporation of DCM, without any further treatment (recovered product: 9.9 g; 63% yield).. The final product appears as a transparent viscous liquid and was used for the subsequent study of aza-Michael addition of primary amines.

Enzymatic Synthesis of Poly (1,4-Cyclohexanedimethanol Itaconate) Catalyzed by CaLB Covalently Immobilized on Epoxy Methacrylic Resins (135 U per g of Monomers)

The reaction was carried out using DMI (0.044 mol, 7 g) and BDO (0.0484 mol, 6.9798 g) in molar ratio 1:1.1 and adding 40% w/w of immobilized CaLB on Relizyme® EC-EP (135 U/g monomers). The thin-film enzymatic polycondensation[15] was carried in a 250 mL round-bottom flask connected to a rotary evaporator at 50 °C and 70 mBar. After 72 h, the reaction mixture was recovered with DCM and products were characterized after filtration of the biocatalyst and evaporation of DCM, without any further treatment (recovered product: 8.68 g; 62% yield). The final product appears as a yellowish transparent viscous liquid and was used for the subsequent study of the aza-Michael addition of primary amines.

Reaction Between Dimethyl Adipate and Hexanemethylenediamine

The reaction was carried out for 20 h in a closed 2 mL vial at 50 °C at atmospheric pressure while stirring. 4.8 mmol of dimethyl adipate (DMA) were mixed to 0.96 mmol of hexanemethylenediamine (HMDA) in a molar ratio of 1:0.2. Aliquots were taken for monitoring the reaction at 3 h and 20 h and analysed without any further purification.

Reaction Between Dimethyl Itaconate and Hexanemethylenediamine

The reaction was carried out for 20 h in a closed 2 mL vial at 50 °C at atmospheric pressure in dark while stirring. 0.0048 mol of dimethyl itaconate (DMI) were mixed to 0.96 mmol of hexanemethylenediamine (HMDA) in a molar ratio of 1:0.2. Aliquots were taken for monitoring the reaction at 3 min, 90 min, 3 h and 20 h and analyzed without any further purification.

Aza-Michael Addition of Hexamethylenediamine to Poly(1,4-Butylene Itaconate) Oligomers

The first aliquot of anhydrous HMDA (0.00324 mol, 0.3765 g, 20% in comparison to the presence of DMI monomers in the oligomer) was weighed in a closed 100 mL round bottom flask at atmospheric pressure and 40 °C, temperature that enables the complete melting of HMDA. 3.5 g of PBI oligomers (0.0162 mol of DMI monomers) were then added while continuously stirring. This addition indicates the start of the reaction. The molar ratio between the amine added under these conditions and the DMI monomers reached 0.6:1 by adding other two amine aliquots of 20%. The reaction was stopped after 456 min (7 hours and 45 min) by transferring the flask at −20 °C. The samples at different reaction times were taken without any further purification to monitor the reaction course.

Aza-Michael Addition of Phenylethylamine to Poly (1,4-Butylene Itaconate) Oligomers

2 g of PBI oligomers (0.00925 mol of DMI monomers) were weighed in a closed 100 mL round bottom flask at atmospheric pressure and 50 °C. The first aliquot of anhydrous PEA (1.85 mmol, 0.2249 g, 20% in comparison to the presence of DMI monomers in the oligomer) was then added while continuously stirring. Please, notice that PEA is a liquid. This addition indicates the start of the reaction. The molar ratio between the amine added under these conditions and the DMI monomers reached 0.6:1 by adding other two amine aliquots of 20%. The reaction was stopped after 456 min (7 hours and 45 min) by transferring the flask at −20 °C. The reaction mixture appears as a transparent orange liquid because of the presence of the orange amine. The samples at different reaction times were taken without any further purification to monitor the reaction course.

Aza-Michael Addition of Hexamethylenediamine to Poly(1,4-Cyclohexanedimethanol Itaconate) Oligomers

3.5 g of PCI oligomers (0.013 mol of DMI monomers) were first weighed in a 100 mL round bottom flask at atmospheric pressure and warmed at 40 °C, temperature that enables the complete melting of HMDA. The first aliquot of anhydrous HMDA (0.0026 mol, 0.3009 g, 20% in comparison to the presence of DMI monomers in the oligomer) was then added while continuously stirring. This addition indicates the start of the reaction. The molar ratio between the amine added under these conditions and the DMI monomers reached 0.6:1 by

adding other two amine aliquots of 20%. The reaction was stopped after 456 min (7 hours and 45 min) by transferring the flask at −20 °C. The reaction mixture appears as a milky viscous liquid with white solid unreacted particles of HMDA. The samples at different reaction times were taken without any further purification to monitor the reaction course.

Aza-Michael Addition of Hexamethylenediamine to Poly(1,4-Cyclohexanedimethanol Itaconate) Oligomers in DCM

1.16 g of PCI oligomers (corresponding to 0.013 mol of DMI monomers) were dissolved in 20 mL of DCM in a closed 100 mL round bottom flask at atmospheric pressure and 40 °C. The first aliquot of anhydrous HMDA (0.88 mmol, 0.103 g, 20% in comparison to the presence of DMI monomers in the oligomer) was then added while continuously stirring. This addition indicates the start of the reaction. The molar ratio between the amine added under these conditions and the DMI monomers reached 0.6:1 by adding other two amine aliquots of 20%. The reaction was stopped after 456 min (7 hours and 45 min) by transferring the flask at −20 °C. The reaction mixture appears as a transparent liquid. The samples at different reaction times were taken without any further purification to monitor the reaction course.

Aza-Michael Addition of Phenylethylamine to Poly (1,4-Cyclohexanedimethanol Itaconate) Oligomers

3.5 g of PCI oligomers (0.013 mol of DMI monomers) were first weighed in a closed 100 mL round bottom flask at atmospheric pressure and 50 °C. The first aliquot of anhydrous PEA (0.0026 mol, 0.3138 g, 20% in comparison to the presence of DMI monomers in the oligomer) was then added while continuously stirring. Please, notice that PEA is a liquid. This addition indicates the start of the reaction. The addition order of amine and PCI oligomers has been inverted compared to the procedures with PBI oligomers, to ensure a more reliable reaction sample at time 0 min.

The molar ratio between the amine added under these conditions and the DMI monomers reached 0.6:1 by adding other two amine aliquots of 20%. The reaction was stopped after 456 min (7 hours and 45 min) by transferring the flask at −20 °C. The reaction mixture appears as a transparent orange liquid because of the presence of the orange amine. The samples at different reaction times were taken without any further purification to monitor the reaction course.

Molecular Modelling

The crystal structure of CaLB 1TCA[32] was taken from the Protein Data Bank50 and was prior pre-processed using PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC) with the removal of all water molecules and ligands.[32a] The protonation state was computed at pH 7.0 using the PROPKA-based[33] PDB2PQR online server.[34] Therefore, the pdb structure was defined into the OPLS-AA force-field[35] in the software GROMACS version 4 according to the previously calculated protonation state.[36] Using the TIP4 water model,[37] the structure was inserted in a 343 nm^3 water box, while including sodium and chloride ions at the concentration of 0.1 M in the correct proportion to neutralize the charge of the system. A minimization step preceded the Molecular Dynamic (MD) simulation and was performed with 10000 iterations of a steepest descendent algorithm in GROMACS version 4. The actual MD simulation took place for 10 ns at 45 °C in an NVT environment using Particle Mesh Ewald (PME) algorithm[38] for the calculation of electrostatic interactions and keeping the temperature and pressure constant (v-rescale algorithm[39] for temperature and Berendsen algorithm[40] for pressure). The proper protein structure used for the docking calculations was identified using the http://manual.gromacs.org/archive/4.6.1/online/g_cluster.html tool of GROMACS version 4 to select the more representative conformation in the trajectory file, discarding the first half of the MD simulation to allow the equilibration of the protein in the solvent.

For the docking investigation of the substrates, AutoDock version 4.2 was used.[41] The two conformers were processed using a Lamarckian genetic algorithm that was run 100 times, each one comprising 250 docking poses. Eventually the best 100 poses were selected according to AutoDock scoring function based on binding energy. A visual inspection of the poses allowed to extract the Near Attack Conformation (NAC) poses corresponding to the productive ones. NACs are defined as conformations compatible with the attack of the catalytic serine to the electrophilic carbon of the acyl group.[42]

Acknowledgements

This work was financially supported by MIUR trough Università degli Studi di Trieste (FRA 2018). The authors acknowledge Simone Lotteria for the covalent immobilization of CaLB on rice husk, Fabio Hollan for the mass spectrometry analysis and Riseria Cusaro (Binasco, Italy) for the samples of the rice husk.

References

[1] a) UN Environment Annual Report 2017. https://www.unenvironment.org/annualreport/2017/index.php?page=0&lang=en. (Accessed: 18th July 2018); b) Ambitious new strategy to make plastic fantastic https://ec.europa.eu/environment/efe/themes/economics-strategy-and-information/ambitious-new-strategy-make-plastic-fantastic_en. (Accessed: 18th July 2018); c) A. Pellis, E. Herrero Acero, V. Ferrario, D. Ribitsch, G. M. Guebitz, L. Gardossi, *Trends Biotechnol.* **2016**, *34*, 316–328; d) T. J. Farmer, M. Mascal, *Platform Molecules. In Introduction to Chemicals from Biomass* (Eds.: J. Clark, F. Deswarte), John Wiley & Sons Ltd, **2015**, pp 89–155.

[2] A. Pellis, E. Herrero Acero, L. Gardossi, V. Ferrario, G. M. Guebitz, *Polym. Int.* **2016**, *65*, 861–871.

[3] a) T. J. Farmer, J. W. Comerford, A. Pellis, T. Robert, *Polym. Int.* **2018**, *67*, 775–789; b) J. Trotta, A. Watts, A. Wong, A. M. La Pointe, M. A. Hillmyer, B. P. Fors, *ACS Sustainable Chem. Eng.* **2019**, *7*, 2691–2701; c) G.

Giacobazzi, C. Gioia, M. Colonna, A. Celli, *ACS Sustainable Chem. Eng.* **2019**, *7*, 5553–5559.
[4] P. Spasojevic, M. Zrilic, V. Panic, D. Stamenkovic, S. Seslija, S. Velickovic, *Int. J. Polym. Sci.* **2015**, *2015*, 1–9.
[5] K. Satoh, D.-H. Lee, K. Nagai, M. Kamigaito, *Macromol. Rapid Commun.* **2014**, *35*, 161–167.
[6] R. Coşkun, A. Delibaş, *Polym. Bull.* **2012**, *68*, 1889–1903.
[7] a) I. Schoon, M. Kluge, S. Eschig, T. Robert, *Polymers* **2017**, *9*, 693; b) T. J. Farmer, R. Castle, J. Clark, D. Macquarrie, *Int. J. Mol. Sci.* **2015**, *16*, 14912–14932; c) M. Sakai, *Bull. Chem. Soc. Jpn.* **1976**, *49*, 219–223; d) D. G. Barrett, T. J. Merkel, J. C. Luft, M. N. Yousaf, *Macromolecules* **2010**, *43*, 9660–9667.
[8] a) A. Pellis, L. Corici, L. Sinigoi, N. D'Amelio, D. Fattor, V. Ferrario, C. Ebert, L. Gardossi, *Green Chem.* **2015**, *17*, 1756–1766; b) L. Corici, A. Pellis, V. Ferrario, C. Ebert, S. Cantone, L. Gardossi, *Adv. Synth. Catal.* **2015**, *357*, 1763–1774.
[9] a) Y. Jiang, A. J. J. Woortman, G. O. R. A. Ekenstein, D. M. Petrovic', K. Loos, *Biomacromolecules* **2014**, *15*, 2482–2493; b) Y. Jiang, A. J. J. Woortman, G. O. R. A. van Ekenstein, K. Loos, *Biomolecules* **2013**, *3*, 461–480; c) Y. Jiang, A. J. J. Woortman, G. O. R. A. van Ekenstein, K. Loos, *Polym. Chem.* **2015**, *6*, 5451–5463.
[10] A. Pellis, P. A. Hanson, J. W. Comerford, J. H. Clark, T. J. Farmer, *Polym. Chem.* **2019**, *10*, 843–851.
[11] a) A. Y. Rulev, *Russ. Chem. Rev.* **2011**, *80*, 197–218; b) R. Ballini, G. Bosica, D. Fiorini, A. Palmieri, M. Petrini, *Chem. Rev.* **2005**, *105*, 933–972.
[12] a) A. Y. Rulev, H. Kotsuki, J. Maddaluno, *Green Chem.* **2012**, *14*, 503–508; b) G. Bosica, A. J. Debono, *Tetrahedron* **2014**, *70*, 6607–6612; c) S. Strompen, M. Weiß, T. Ingram, I. Smirnova, H. Gröger, L. Hilterhaus, A. Liese, *Biotechnol. Bioeng.* **2012**, *109*, 1479–1489.
[13] a) T. P. Culbertson, J. M. Domagala, T. F. Mich, J. B. Nichols, *J. Med. Chem.* **1987**, *10*, 1711–1715; b) F. Felluga, G. Pitacco, M. Prodan, S. Pricl, M. Visintin, E. Valentin, *Tetrahedron: Asymmetry* **2001**, *12*, 3241–3249; c) B. M. Kenda, A. C. Matagne, P. E. Talaga, P. M. Pasau, E. Differding, B. I. Lallemand, A. M. Frycia, F. G. Moureau, H. V. Klitgaard, M. R. Gillard, B. Fuks, P. Michel, *J. Med. Chem.* **2004**, *47*, 530–549.
[14] M. A. Ali, S. Tateyama, Y. Oka, D. Kaneko, M. K. Okajima, T. Kaneko, *Macromolecules* **2013**, *46*, 3719–3725.
[15] a) T. J. Farmer, D. J. Macquarrie, J. W. Comerford, A. Pellis, J. H. Clark, *J. Polym. Sci., Part A: Polym. Chem.* **2018**, *56*, 1935–1945; b) S. Chanda, S. Ramakrishnan, *Polym. Chem.* **2015**, *6*, 2108–2114; c) C. Hoffmann, M. C. Stuparu, A. Daugaard, A. Khan, *J. Polym. Sci., Part A: Polym. Chem.* **2015**, *53*, 745–749.
[16] A. Lv, Y. Cui, F.-S. Du, Z.-C. Li, *Macromolecules* **2016**, *49*, 8449–8458.
[17] B. C. Ranu, S. Banerjee, *Org. Lett.* **2005**, *7*, 3049–3052.
[18] a) B. F. Barton, S. L. Cook, J. S. Howell, N. G. Mc Millian, D. B. Shackelford, B. A. Tennant, P. W. Turner, *World Patent* WO 2013019441, **2013**; b) D. I. Collias, A. M. Harris, V. Nagpal, I. W. Cottrell, M. W. Schultheis, *Ind. Biotechnol.* **2014**, *10*, 91–105.
[19] X. Wang, Q. Wang, S. Liu, G. Wang, *Polym. Degrad. Stab.* **2018**, *154*, 96–102.
[20] a) Y. Jiang, G. O. R. A. van Ekenstein, A. J. J. Woortman, K. Loos, *Macromol. Chem. Phys.* **2014**, *215*, 2185–2197; b) F. Binns, P. Harffey, S. M. Roberts, A. Taylor, *J. Chem. Soc.*, *Perkin Trans. 1* **1999**, 2671–2676.
[21] L. Corici, V. Ferrario, A. Pellis, C. Ebert, S. Lotteria, S. Cantone, D. Voinovich, L. Gardossi, *RSC Adv.* **2016**, *6*, 63256–63270.
[22] A. Pellis, V. Ferrario, M. Cespugli, L. Corici, B. Zartl, E. Herrero Acero, C. Ebert, G. M. Guebitz, L. Gardossi, *Green Chem.* **2017**, *19*, 490–502.
[23] M. B. Ansorge-Schumacher, O. Thum, *Chem. Soc. Rev.* **2013**, *42*, 6475–6490.
[24] G. Cerea, L. Gardossi, L. Sinigoi, D. Fattor, *World Patent* WO2013110446A1, **2013**.
[25] T. C. Bruice, F. C. Lightstone, *Acc. Chem. Res.* **1999**, *32*, 127–136.
[26] S. Kim, C. Jiménez-González, B. E. Dale, *Int. J. Life Cycle Assess.* **2009**, *14*, 392–400.
[27] M. Cespugli, S. Lotteria, L. Navarini, V. Lonzarich, L. Del Terra, F. Vita, M. Zweyer, G. Baldini, V. Ferrario, C. Ebert, L. Gardossi, *Catalysts* **2018**, *8*, 471.
[28] M. Sakai, S. Nishikawa, H. Koike, Y. Sakakibara, N. Uchino, *Bull. Chem. Soc. Jpn.* **1978**, *51*, 2970–2972.
[29] G. Bosica, R. Abdilla, *Molecules* **2016**, *21*, 815.
[30] M. Vastano, A. Pellis, B. Immirzi, G. Dal Poggetto, M. Malinconico, G. Sannia, G. M. Guebitz, C. Pezzella, *Green Chem.* **2017**, *19*, 5494–5504.
[31] T. Robert, S. Friebel, *Green Chem.* **2016**, *18*, 2922–2934.
[32] a) H. M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T. N. Bhat, H. Weissig, I. N. Shindyalov, P. E. Bourne, *Nucleic Acids Res.* **2000**, *28*, 235–242; b) J. Uppenberg, M. T. Hansen, S. Patkar, T. A. Jones, *Structure* **1994**, *2*, 293–308.
[33] C. R. Sondergaard, M. H. M. Olsson, M. Rostkowski, J. H. Jensen, *J. Chem. Theory Comput.* **2011**, *7*, 2284–2295.
[34] T. J. Dolinsky, J. E. Nielsen, J. A. McCammon, N. A. Baker, *Nucleic Acids Res.* **2004**, *32*, W665–W667.
[35] G. A. Kaminski, R. A. Friesner, J. Tirado-Rives, W. L. Jorgensen, *J. Phys. Chem. B* **2001**, *105*, 6474–6487.
[36] B. Hess, C. Kutzner, D. van der Spoel, E. Lindahl, *J. Chem. Theory Comput.* **2008**, *4*, 435–447.
[37] H. W. Horn, W. C. Swope, J. W. Pitera, J. D. Madura, T. J. Dick, G. L. Hura, T. Head-Gordon, *J. Chem. Phys.* **2004**, *120*, 9665–9678.
[38] U. Essmann, L. Perera, M. L. Berkowitz, T. Darden, H. Lee, L. G. Pedersen, *J. Chem. Phys.* **1995**, *103*, 8577–8592.
[39] G. Bussi, D. Donadio, M. Parrinello, *J. Chem. Phys.* **2007**, *126*, 014101.

[40] H. J. C. Berendsen, J. P. M. Postma, W. F. van Gunsteren, A. Di Nola, J. R. Haak, *J. Chem. Phys.* **1984**, *81*, 3684–3690.

[41] a) G. M. Morris, R. Huey, W. Lindstrom, M. F. Sanner, R. K. Belew, D. S. Goodsell, A. J. Olson, *J. Comput. Chem.* **2009**, *30*, 2785–2791; b) G. M. Morris, D. S. Goodsell, O. S. Halliday, R. Huey, W. E. Hart, R. K. Belew, A. J. Olson, *J. Comput. Chem.* **1998**, *19*, 1639–1662.

[42] T. C. Bruice, F. C. Lightstone, *Acc. Chem. Res.* **1999**, *32*, 127–136.