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Variation in structure and properties of poly(glycerol adipate) via control of chain branching during enzymatic synthesis

V. Taresco [a], R.G. Creasey [a], J. Kennon [b], G. Mantovani [a], C. Alexander [a], J.C. Burley [a], M.C. Garnett [a, *]

[a] University of Nottingham, School of Pharmacy, University Park, Nottingham, NG7 2RD, UK
[b] University of Nottingham, School of Chemistry, University Park, Nottingham, NG7 2RD, UK

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ABSTRACT

Poly (glycerol adipate) (PGA) can be produced from divinyl adipate and unprotected glycerol by an enzymatic route to generate a polymer with relatively low molar mass (12 kDa). PGA bears a pendant hydroxyl group which imparts a hydrophilic character to this water insoluble polymer. We have examined the effect of synthesis temperature on polymer characteristics through various techniques including FT-IR, ^{1}H and ^{13}C NMR, surface and thermal analysis, both to expand the data already present in the literature about this material and to understand better its properties for potential pharmaceutical applications. The use of a lipase (Novozym 435) as a catalyst suppresses cross-linking at the pendant glyceryl hydroxyl through steric hindrance at the active site, thus producing polymers with low degrees of branching (5–30%), and removes the need for any pre- or post-polymerization protection/deprotection reactions. Careful temperature control during synthesis can give polymers with reproducible molecular weights and reduced amounts of polymer branching compared to synthesis at higher temperatures. Due to the ability of the synthetic route to produce a range of structures, PGA generated by enzymatic routes may emerge as a useful biodegradable polymer platform to engineer solid dispersions or nanoparticles for healthcare applications.

1. Introduction

In recent years advances in polymer science have led to significant improvements in a plethora of fields, from electronics to healthcare, pharmacy and packaging [1,2]. In nanomedicine, polymers can be functionalized with bioactive molecules and processed in a range of shapes and sizes to address specific *in vivo* requirements. The increasing need for more sophisticated materials in various applications requires more versatility via functional macromolecules [3]. For most applications in nanomedicine, biodegradable polymers are necessary for regulatory reasons and these degradation properties can impart additional functional behavior. Poly(ortho esters) are attractive because of their inherent hydrolytic instability but poly(ortho esters) with valuable functional utility are difficult to produce because of complex monomer production, protection deprotection strategies or polymer degradation during protection/deprotection [4].

Synthetic aliphatic polyesters are widely used for biomedical, pharmaceutical and environmental applications due to their high biodegradability and low cost of production. However, the standard synthetic path for production of medical polyesters uses a metal catalyst and high temperatures (normally above 120 °C). In these conditions a wide range of collateral reactions can occur and residual toxic metals can remain entrapped in the final material. Additionally, most current metal-based catalysts lack stereo- and regio-selectivity. Over the last three decades enzymatic polymerization has been developed, becoming an important route to produce new synthetic polymers [5]. A very fertile area of research for tailored polymer synthesis is the use of enzymes in organic solvents to generate polymers with highly specific physico-chemical properties [6]. Thus lipase/esterase-catalyzed polycondensations have been developed as a suitable alternative to metal-based catalysis strategies in order to direct formation of desirable polymer structures [3,5,7–10]. Advantages of enzymatic polymerization include (a) mild reaction conditions, (b) excellent control of enantio-, chemo-, and regioselectivity, (c) ability to catalyze the ring-opening

* Corresponding author.
E-mail address: martin.garnett@nottingham.ac.uk (M.C. Garnett).

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polymerization of macrocyclic lactones, avoiding protection/deprotection steps, (d) low toxicity and often recyclability of biocatalysts, and (e) tunable catalytic activity, with few by-products [3,5,6,9,11,12]. Hence, enzymatic polymerization is emerging as a 'green' and more selective route than traditional ones to obtain well-defined polyesters [13,14].

Enzymatic polyester synthesis can be divided into two major categories: (I) ring-opening polymerization of cyclic monomers, *i.e.* lactones or lactams [15] and (II) polycondensation of diacids with polyalcohols [16]. Enzymatic polymerizations are routinely carried out either in organic solvents or in bulk (solvent free). The enzyme is often immobilized on a solid support (such as acrylic beads, porous silica particles, etc) [11] in order to maintain activity of the biological catalyst and facilitate separation of the catalyst from the final polyester polymers. Lipases are effective catalysts for the synthesis of polyesters [6,17]. Yang et al. were the first to synthesize a family of aliphatic polyesters consisting of diesters, diols and glycolate repeat units through the copolymerization of ethyl glycolate with diethyl sebacate and 1,4-butanediol [18]. Liu et al. synthesized a series of biodegradable poly(amine-co-esters) via one step enzymatic copolymerization of diesters with C_4–C_{12} chain length and diethanolamine or its derivatives [11]. A really promising area is the enzymatic (trans)esterification of polyalcohols and di-acids (or di-esters). Iglesias et al. [19] used glycerol and adipic acid to produce hydroxylated polyesters. Partial selectivity towards the formation of 1,3 disubstituted glycerol repeating units was achieved due to the higher reactivity of lipase towards primary alcohols compared to secondary hydroxyl groups [19]. Novozym 435 (immobilized *Candida antarctica* lipase B) is the most widely used lipase enzyme utilized for the synthesis of polyesters due to its temperature and organic solvent tolerance, and high regio- chemo- and stereo-selectivity [8,20].

Kline et al. [21] first reported the synthesis of poly(glycerol adipate) (PGA) from glycerol and divinyl adipate using Novozym 435. Vinyl esters are ideal monomers for this process as the transesterification co-product, vinyl alcohol, readily tautomerizes to acetaldehyde, which is then no longer available as a substrate. This accordingly eliminates the undesired transesterification cleavage of the polyester backbone which would otherwise prevent the formation of high molecular weight polymers [16]. To avoid the more expensive vinyl ester, syntheses using unmodified adipic acid or the dimethyl ester have also been reported, but these generally require higher temperatures and carrying out the reaction under vacuum to achieve similar efficiency of polymerization [22,23]. Novozym 435 has been widely used for polymerizations of this type due to its intrinsic resistance to acetaldehyde compared to other enzymes. Work by Korupp et al. [22] has shown that optimizing the reaction conditions - temperature, pressure, enzyme concentration, reactants ratio, stirrer type, stirring rate and reaction time - enables synthesis of PGA up to a 500 g scale with ~95% monomer conversion and molecular weights (absolute molecular weight) of 2–3 kDa. By further optimizing reaction parameters such as temperature, feed ratio between monomers, and enzyme origin, Uyama et al. [24] achieved regioselective control in the lipase-catalyzed polymerization of divinyl sebacate and various triols. Yang et al. [25] compared synthesis conditions and structure of poly(oleic diacid-co-glycerol) resulting from the use of Novozym 435 or dibutyl tin oxide (DBTO) as catalysts. In the first case, a polyester with low branching degree was obtained while in the metal-catalyzed synthesis a gel was formed due to extensive cross-linking. Kallinteri et al. [26] reported the optimization of the enzymatic synthesis of PGA, mainly through control of water content, use of solvent and increased reaction time to obtain various molecular weights and demonstrated the incorporation of various amounts of different acyl substituents through subsequent modification.

PGA polymers and side-chain acylated derivatives have also been shown to self-assemble into nanoparticles with the ability to entrap a drug, dexamethasone phosphate, with increased efficiency dependent on M_w and degree of acylation of residual hydroxyls on the polymer backbone [26]. Conversely, the polar anticancer drug cytosine arabinoside showed maximum loading and slowest release from the parent unsubstituted PGA polymer with the highest molecular weight (12 kDa) [27]. Thus functionalization has the potential to provide a wide range of polymer properties which could be developed for a variety of applications. This new family of nanoparticles offers properties vital to lipophilic drug administration, such as the absence of any emulsifier or stabilizer and increased stability [28].

Both the unmodified PGA and acyl substituted PGA have been shown to have low toxicity on HL-60 and HepG2 cell lines [26], and the unmodified PGA was well tolerated in a chronic oral dosing study (No Observed Adverse Effect Level in rats determined at 1000 mg/kg/day, data not shown). Collectively these polymer properties are of great interest for drug delivery studies.

If these enzymatically synthesized polymers are to be useful for future drug delivery applications, their synthesis needs to be reproducible, convenient, able to produce polymers of high molecular weight, with well defined structures. Previous literature, particularly for polyglycerol adipate, covered a wide variety of different reaction conditions which have been employed by various authors and in addition, a wide range of temperatures up to 90 °C. The resulting polymers were often incompletely characterized. We have in this paper compared the effect of using different synthesis temperatures on the resulting physicochemical properties of PGA to establish optimum synthesis conditions. We have examined physico-chemical properties of PGA, such as Tg and contact angle. Different techniques including FT-IR, ^{1}H and ^{13}C NMR, surface and thermal analysis have been carried out to achieve a better insight of the features and behavior of these materials thereby clarifying the potential of PGA in novel drug delivery applications.

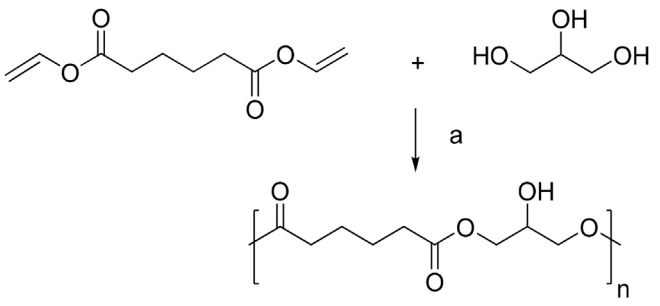
2. Experimental section

2.1. Materials

Novozym 435 lipase ([9001-62-1], derived from *C. antarctica* (>5000 U/g) and immobilized on an acrylic macroporous resin, and all solvents were purchased from Sigma–Aldrich. Divinyl adipate [4074-90-2] and HOPG (Highly Ordered Pyrolytic Graphite) were purchased from TCI America and SPI supplies, respectively. All chemicals were used as received.

2.2. Synthesis of Poly(glycerol adipate) in the range of temperature from 40 to 70 °C

PGA was synthesized (Scheme 1) by enzymatic polymerization



Scheme 1. Reagents and conditions: a. Novozym 435, 50 (60, or 70) °C, THF, 24 h.

of divinyl adipate (DVA) and glycerol following a protocol adapted from Kallinteri et al. [26].

Glycerol and DVA (125 mmol of each) were poured into a 250 ml 3-neck round bottomed flask and dissolved in anhydrous THF (50 ml) in presence of Novozym 435 (1.1 g). The resulting mixture was stirred at 250 rpm using an overhead stirrer fitted with a Teflon bladed paddle for 24 h at 40, 50, 60 or 70 C.

The reaction was stopped by filtration of the immobilized enzyme, followed by evaporation of the solvent under reduced pressure. The resulting residue was then heated at 90–95 °C for 1 h to deactivate any residual enzyme which may have leached out of the resin. This step is vital to reduce polymer hydrolytic side reactions that can occur when traces of active lipase are left in the final polymer [29]. The residue was kept under vacuum at 40 C for three days to remove residual THF, until a stable mass was reached. The resultant material was a highly viscous yellow liquid, which exhibit no detectable traces of solvent or monomer in its ^{1}H NMR spectrum.

2.3. Characterization techniques

2.3.1. Chemical structure identification

FT-IR spectra were recorded with an Attenuated Total Reflection spectrophotometer (Agilent Technologies Cary 630 FTIR) equipped with a diamond single reflection ATR unit. Spectra were acquired with a resolution of 4 cm^{-1}, in the range 4000-650 cm^{-1} by recording 32 interferograms.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer using acetone-d_6 as the solvent. Chemical shifts are expressed in parts per million (δ) downfield from internal standard tetramethylsilane.

2.3.2. Molecular weight

Gel permeation chromatography (GPC) was carried out by using a PL50 Polymer Laboratories system, employing 2 mixed bed (D) columns at 50 °C, using DMF + 0.1% LiCl as the mobile phase, flow rate 1 mL min^{-1}, equipped with a refractive index detector. Poly(-methyl methacrylate) standards (M_n range: 1,810,000–505 g mol^{-1}) were used to calibrate the SEC.

2.3.3. Physical characterization

Contact angles values were measured at 25 °C using a KSV Cam 200 (KSV Instruments Ltd., Helsinki, Finland) equipped with dedicated software (CAM200). Samples were prepared by coating glass microscope slides with polymer thin films by solvent evaporation from 3% w/w solutions of polymers in acetone. Measurements were carried out as follows: polymer-coated slides were mounted horizontally on the stand of the instrument. A small drop of distilled water was dispensed by a syringe with a flattened tip needle onto the polymer film. The tangent line was recorded using a camera and both shape and contact angle were analyzed. Four measurements were recorded for each polymer.

Polymer thermal properties were investigated by differential scanning calorimetry (DSC) (Q2000, TA Instruments, Leatherhead, UK) at a heating rate of 10 °C/min. Thermal Analysis Software (Version 4.5.05A) was used to analyze the resulting data. Pans with pin holed lids (TA Instruments, Brussels, Belgium) were utilized for the analysis of the samples, using empty pans as the reference. The DSC cell was purged with nitrogen gas at a flow rate of 50 mL/min. Glass transitions were analyzed performing two heating/cooling cycles from −80 up to 120 °C.

Atomic force microscopy (AFM) measurements were performed using a Bruker Icon FastScan SPM in PeakForce Quantitative Nanomechanical Mapping (QNM) mode using NFESPA probes (nominal f = 75 kHz, k = 3 N/m). Samples for AFM measurements were prepared by dissolving polymers in acetone followed by spin-coating 5 μL of the resulting solutions onto freshly cleaved mica or HOPG at 2000 rpm. Images shown in this study are from 0.1 mg mL^{-1} solutions.

3. Results and discussion

3.1. Chemical structure and molecular weight

PGA homopolymer was soluble in a wide range of organic solvents, indicating that no significant chain–chain crosslinking occurred in the polycondensation step. Condensation of carboxylic acid or their derivatives can occur at both primary and secondary hydroxyl functionalities of glycerol co-monomer, which in polycondensation processes can lead to chain branching [30].

PGA formation was confirmed by 1H NMR (full spectrum Fig. 1SI), which showed complete disappearance of the DVA vinyl proton signals at 7.29, 4.87 and 4.59 ppm and appearance of the PGA pattern of methylene and methine signals (Fig. 1). It is possible that some terminal vinyl group may remain at the end of the reaction. However, we dilute the reaction with THF (not-dried) and heat the polymer to 100 °C to inactivate any residual detached enzyme. We would expect this to promote the hydrolysis of the terminal vinylester, and indeed no evidence of it is present in the NMR.

As expected, all protons related to glyceride repeating units were found between 3.5 and 4.5 ppm. Peaks found in the 5.0–5.5 ppm region revealed the presence of secondary ester moieties, indicating that a small proportion of glycerol secondary hydroxyl groups took part in the polycondensation process. The peak at 5.31 ppm (c'' in Fig. 1 and Fig. 1 inset) corresponds to the methine proton of trisubstituted glycerol units and was used here to calculate the degree of branching of the PGA polymers synthesized. The amount of trisubstituted units was calculated [23] by comparing the integrals of the methine C*H* peak of trisubstituted glycerol at 5.31 ppm, to that at 1.66 ppm of $CH_2CH_2C(O)$ of adipic acid repeating units - c'' and b peaks, respectively, normalizing by the C*H* peak of the terminal group at 3.86 (Fig. 1), and found to be between 5 and 10% mol/mol for polymerizations run at 50 °C for 24 h (see Table 2 in supplementary information). To evaluate the effect of temperature on the degree of branching, polymerization of glycerol and DVA was repeated at 40, 60 and 70 °C. As expected, with an increase in the reaction temperature, an increase in the degree of branching - from 5 up to ~30% - was observed (Fig. 1, inset), while decreasing the temperature of reaction resulted in no apparent change in trisubstituted units (Fig. 1, inset). However, the c' peak, related to 1,2-disubstitution, remained constant at all temperatures. Therefore, increasing the temperature over 50 °C, and thus the kinetics of the reaction, causes the enzyme to perform 1,2,3- trisubstitution after a few hours, as noted in previous publications [24,31]. Conversely on reducing the temperature, no significant advantage was found (Fig. 2). As a result, the discussion refers to that polymer synthesized at 50 °C but similar data were found for the one synthesized at 40 °C.

The integral of the peak, corresponding to the methine proton of terminal 1-substituted glycerol moieties, was utilized to estimate the degree of polymerization of the PGA polymers prepared in this study:

$$DP = \frac{\int \left(\left(V - C'' \right) + A \right)}{\int \left(V - C'' \right)}$$

where V is the integral of the peak at 3.86 ppm, and A and C″ are the

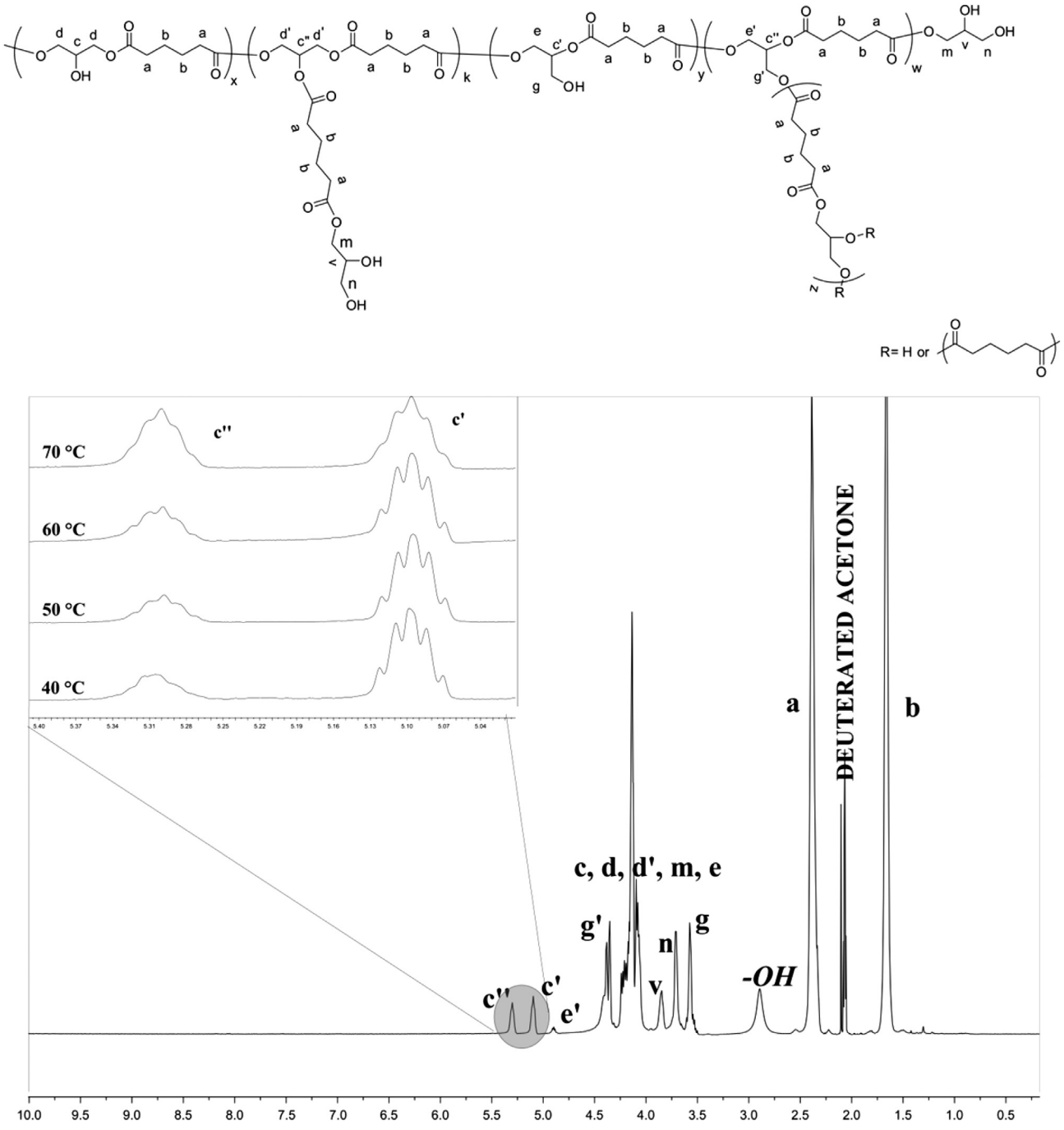


Fig. 1. PGA macromolecular structure with triol-substitution modalities. ^{1}H-NMR spectrum of PGA synthesized at 70 °C. Inset: Effect of reaction temperature on PGA degree of branching.

methylene group within the PGA chain and the methine proton of the trisubstituted unit, respectively. DP was thus calculated to be ~58 units at 40 and 50 °C, similar to values found previously under these conditions [25], and it decreases to ~ 22 as the reaction temperature is increased up to 70 °C. Comparing the observed molecular weight with the DP calculated from NMR shows that the more highly branched polymers have a reduced solvated volume.

Further insights into the nature of PGA macromolecular architecture were gained by ^{13}C NMR (full spectrum Fig. 2SI). This technique was used to qualitatively evaluate the number of methine carbon present along the backbone to investigate the presence of different glyceride groups. Four evident signals of methine CH groups for PGA synthesized at 70 and 60 °C were found in the 65–73 ppm region (Fig. 3), for polymer synthesized at 50 °C the peak was barely visible above baseline [24]. In this latter spectrum the peak related to the 1,2,3-trisubstituted unit is difficult to distinguish its low signal from the noise. (quantitative ^{13}C NMR analysis is ongoing to better establish the amount of trisubstituted units).

Again, the presence of 1,2-disubstituted and 1,2,3-trisubstituted glyceride units in addition to the 1,3-disubstituted and 1-substituted terminal groups was expected for a fully linear

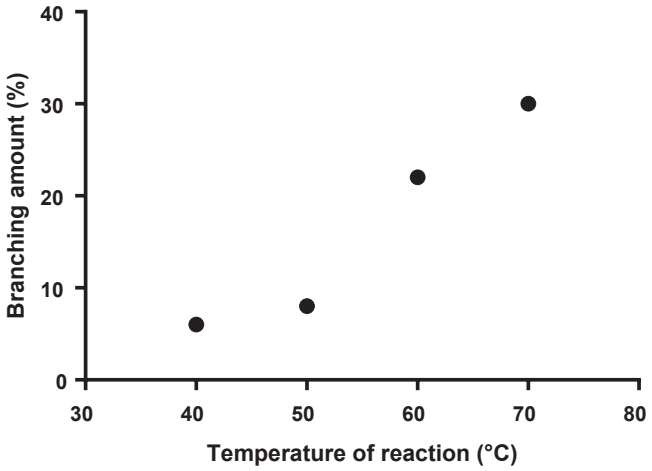


Fig. 2. Polymer branching trend with temperature of reaction.

of reaction, therefore extending the reaction time up to 24 h mainly affects polymer size. A good control of the reaction temperature is key, as Novozym 435 undergoes an important change in conformation at T ≥ 55 °C, and highest activity has been reported to be slightly below the protein denaturation temperature [32]. The presence of the enzyme catalyst is essential as placing the two monomers in THF at either 50 or 70 °C for 3 h resulted in a lack of reaction.

PGA polymer was analyzed by ATR FT-IR (Fig. 4). The spectra for PGAs, synthesized at the four different temperatures, were showing a broad peak, due to hydrogen bonds, for the −OH stretching at 3450 cm^{-1}, symmetric and asymmetric stretching of alkyl C−H bonds between 2950 and 2870 cm^{-1} and 1700 cm^{-1} the stretching of C═O carbonyl group, while at 1380 cm^{-1} CH bending is present and around 1130-1060 cm^{-1} the C−O−C stretching peak appears. No detectable differences in FTIR were apparent at the different synthesis temperatures.

Initial GPC experiments were carried out using THF as the mobile phase, as reported in previous studies [26,28]; however, 1% LiCl DMF at 50 °C was chosen in the present work due to solubility issues and poor reproducibility of the measurements when using THF as the eluent. Polydispersity values (Đ) increased from 2.7 to 4.2 as the temperature of synthesis increased (Table 1).

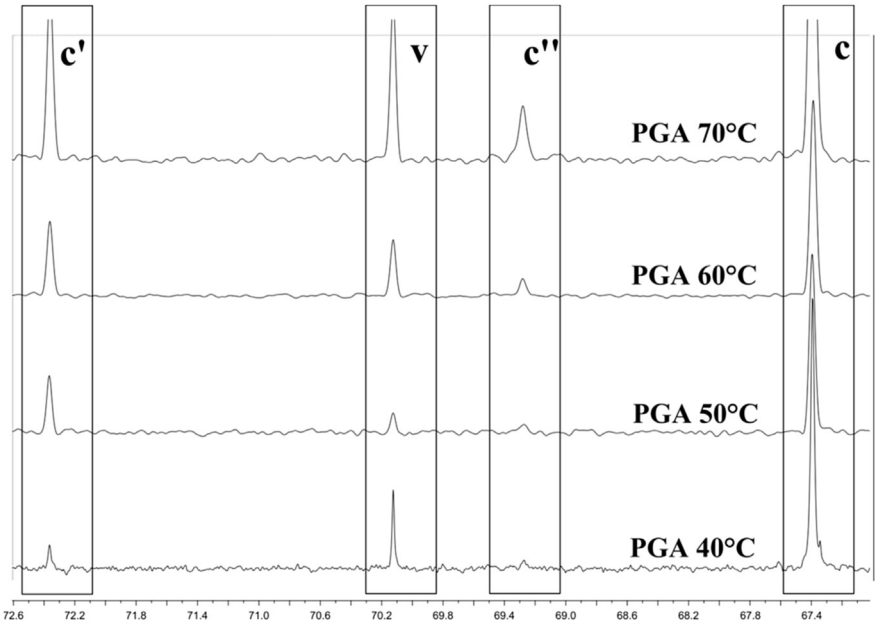


Fig. 3. Partial ^{13}C-NMR spectrum of PGA, showing the signals corresponding to different methine groups of the synthesized PGA polymers.

polymer [31]. The presence of 1,2-disubstituted and 1,2,3-trisubstituted glyceride groups in PGA has been described before [21,24,31], with the latter leading to polymer branching as well as to the decrease of the number of polymer hydroxyl groups. Uyama et al. [24] reported that regioselectivity of lipases towards primary alcohols of triols (leading to linear polymer architectures) was observed only within a specific range of temperatures and reaction times. When preparing PGAs with M_n up to 3.5 kDa, Naolou et al. observed no detectable branching after 9 h of reaction time, working below 60 °C [31]. Kallinteri et al. [21] found that by increasing the reaction timing up to 24 h the molecular weight increased up to 12 kDa, but branching was not investigated in that work. For reaction conditions analogous to those chosen in this present study, Russell et al. estimated the selectivity of the condensation reaction to primary hydroxyl groups of glycerol to be in the 90–95% range [21]. According to NMR observations in the present work and data collected in the relevant cited works [23,24], the branching phenomenon is well documented to occur before 9 h

Đ in the 2–3 range are typical for polymers made by polycondensation, in agreement with the Carothers theory [33,34]. Furthermore, due to the high viscosity of the reaction environment and the trace presence of water, lipase may start some hydrolytic degradation that increases with the contact time [29]. Indeed, the Đ values measured were slightly higher than those reported elsewhere for shorter reaction times [31,35].

The average molecular weights observed, especially M_w, were slightly higher than those previously reported in the literature [26]; however, the different experimental conditions chosen in this study for the GPC analysis make a comparison with literature data difficult. Interestingly, the average molecular weight of PGA decreased from 13.0 to 5.2 kDa when the polymerization

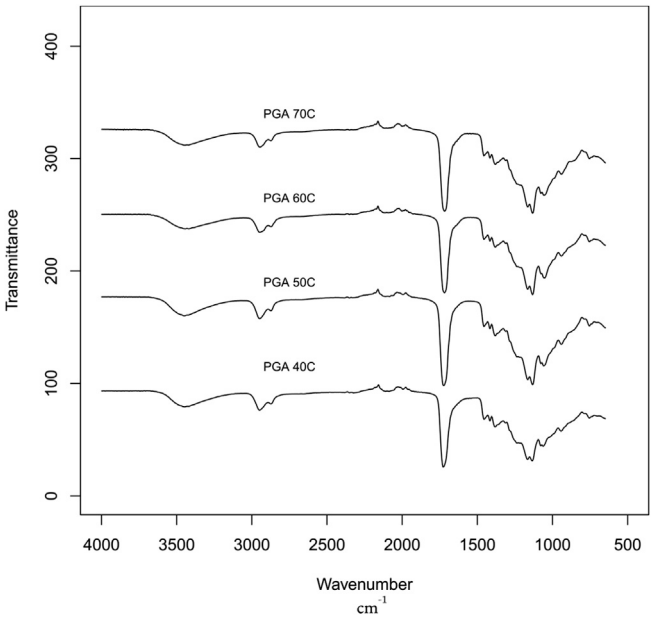


Fig. 4. ATR-IR spectra of PGAs in the spectral range between 4000 and 650 cm^{-1}.

Table 1
Molecular Weights, and Dispersities of polymers utilized in this study.

Polymers	M_n (SEC) kDa	Đ
PGA 70 °C	5.2	4.2
PGA 60 °C	8.9	3.5
PGA 50 °C	13.0	2.8
PGA 40 °C	11.4	2.7

Molecular mass determined by SEC at 50 °C, using DMF + 0.1% LiCl as the mobile phase.

temperature was increased from 50 to 70 °C, respectively, as shown in Table 1. This effect can be ascribed to a number of different phenomena, or a combination thereof. For step-growth polymerizations, such as polycondensations, the polymer molecular weight increases significantly towards the end of the polymerization reaction, where short oligomeric species start to react with each other. It is possible that higher reaction temperatures eventually led to catalyst deactivation, partially preventing this transesterification process [36]. In addition, as demonstrated in this study, at higher temperature the selectivity of the Novozym lipase catalyst for primary glycerol hydroxyl groups is lowered, leading to more branched polymers which possess a smaller apparent molecular weight than equivalent linear polymers and hence a smaller solvated volume. GPC samples were analyzed both before and after filtration and centrifugation and there was no difference between Mn/Mw/D so the change in apparent molecular weight is not due to increased insolubility of the branched polymer in DMF.

3.2. Physical and surface characterization of PGA

Because of the interest in these polymers for potential uses in drug delivery, further physicochemical characterization was carried out to provide underpinning data which would be useful in future studies. Contact angle (Θ) measurements on polymer-coated glass covers were carried out to assess polymer wettability properties. Wettability helps to understand the hydrophilicity/hydrophobicity characteristics which are likely to be important in drug incorporation work. PGA is a completely amorphous material and is able to swell in water without showing water solubility [31]. Water can in principle be absorbed by the polymer via two main mechanisms: (1) physical entrapment within accessible interstices of the polymer and (2) interaction with polymer functional groups via hydrogen bonds. These mechanisms are largely responsible for polymer swelling and cause water accumulation in amorphous and viscoelastic polymers [37]. PGA prepared at 50 °C possesses an experimental Θ in between 58.2° ± 0.9°, while its hydrophobic character increased for polymers prepared at 60 and 70 °C (Θ = 65.0° and 72.0° ± 1.0° respectively), most likely due to the decreased number and accessibility of polymer hydroxyl groups in more branched materials. By increasing the branching amount, hydroxyl free groups are less available due to spatial rearrangement. A branched matrix enhances the hydrophobicity of the material, as decreasing the number of free hydroxyl groups causes the hydrophobic portion to become dominant over the hydrophilic portion.

In a first attempt to better understand the surface wettability properties, PGA synthesized at 70 °C was analyzed by AFM (Fig. 5a, b). Samples deposited by spin coating onto a hydrophilic surface such as mica showed large aggregations of polymer, even when diluted down to 0.01 mg/mL and deposited at 10,000 rpm (data not shown). These aggregates appear island-like without any obvious structure (Fig. 5b). On the more hydrophobic HOPG surface (Fig. 5a), fewer aggregates were observed; however, attempts to image individual polymer strands were unsuccessful as the sample was not sufficiently immobilized on the HOPG. These results are consistent with the amphiphilic nature of PGA, which can adhere to both hydrophilic and hydrophobic surfaces in addition to cohering. Conversely, on a hydrophobic surface, less aggregation occurs suggesting that the polymer has fewer hydrophobic regions available, either because they are strongly involved in supramolecular interactions, or because they are less present in the original structure.

PGA synthesized at 50 °C showed some variation by AFM compared to 70 °C synthesis (Fig. 5c, d). While large aggregates were observed in some areas on the HOPG sample, small fibrous and layered aggregations were also present (Fig. 5c). On mica, similar structures were observed compared to those previously seen for 70 °C PGA; however, the islands were smaller (Fig. 5d). These data are consistent with a polymer with decreased branching in the polymer, leading to smaller aggregates.

AFM tips modified with NH_2 terminal groups, with increased hydrophilicity, interacted very strongly with PGA synthesized at 70 °C (Fig. 5e, f); particularly when the sample was deposited on HOPG. This suggests that polymer hydrophilic groups are oriented away from the HOPG surface compared to the mica surface, as expected for an amphiphilic molecule. As seen in Fig. 6, within the large polymer aggregates, changes in the contrast of the surface property maps are visible which do not correspond to changes in height. These regions have slightly different properties at the surface of the aggregate, leading to a contrast in tip adhesion (Fig. 6c), sample stiffness (Fig. 6d) and deformation (Fig. 6e).

As the material was synthesized at 70 °C, it showed the most branched nature of the polymers reported here, therefore the property contrast may be associated with the branching of the polymer. Another possible explanation for this phenomenon is that the very broad molecular weight distribution (Đ > 2.5) includes molecules with different physical behavior. Therefore, under this weight distribution curve, chains with both different lengths and different degrees of branching (hence chains with different mobility) are expected, leading to changes in the aggregation properties of PGA [36]. These results are preliminary, and work is ongoing in the use of AFM to investigate PGA physical properties

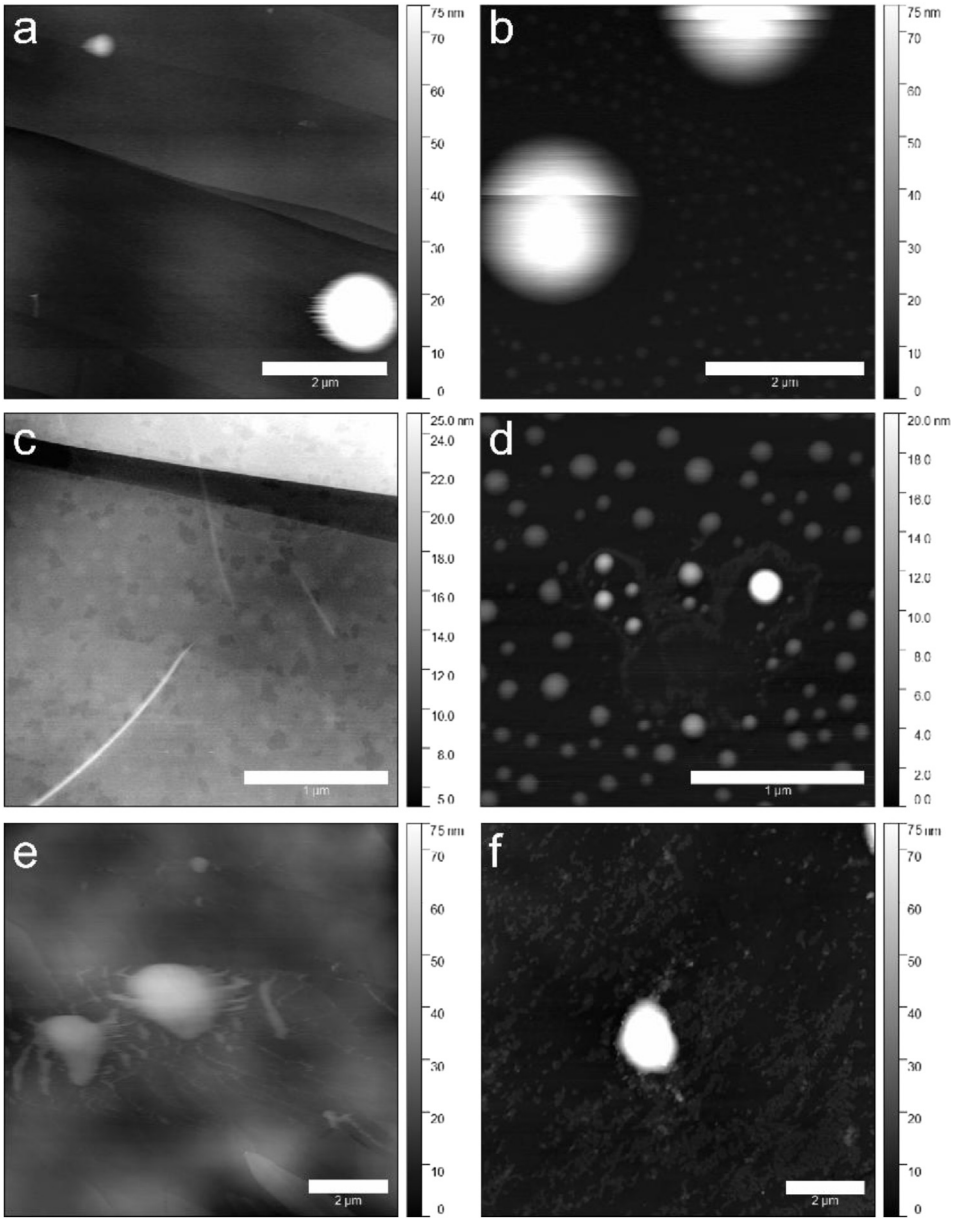


Fig. 5. AFM height images for PGA synthesized at 70 °C (a, b, e, f) and 50 °C (c, d) using a standard Si tip (a, b, e, f) and an APTES-functionalized Si tip (c, d) on hydrophobic HOPG (a, c, e) and hydrophilic mica (b, d, f). Note that (e) is distorted due to excessive interaction between tip and sample. Scale bars are a, b, e, f:2 µm, c, d:1 µm.

relating to the degree of branching and potential phase separation.

PGAs synthesized in this study were also characterized by DSC (Fig. 3SI). T_g values decreased in the range between −49 and −34 °C as the reaction temperature increased. These T_g steps therefore decrease as branching degree increases [38]. From this latter evidence, by merely tuning the reaction temperature it is possible to produce moldable amorphous forms within a certain glass transition temperature range. Presumably the steric hindrance of the branches increases either the flexibility or the free volume of the main backbone.

The synthesis of PGA at 40 and 50 °C was repeated several times in order to evaluate the reproducibility of that procedure. It was found that the amount of trisubstituted units ranged between 5 and 10% while molecular weights were consistent between 10.4 and 13.0 kDa (Table 2 SI).

In summary in this work we showed that by changing only the PGA polymerization temperature, the branching amount varies from 5% up to ~30%. Following and in part optimizing PGA synthetic procedures present in the literature we reached a highly reproducible method that provides a material with a good linearity. Comparing this methodology with the two other main variations in synthesis we can see the advantages and disadvantages of these different methodologies, both from a product specification and green chemistry viewpoint (see Table 3 in supporting information). Our main objectives in optimizing the synthesis have been to maintain a high and reproducible molecular weight suitable for our biomedical applications, with a high conversion rate to minimize the need for purification. Using a lower synthesis temperature is also advantageous in reducing the degree of branching to a minimal level. No other groups have reported achieving the high molecular weight around 25 kDa which we report here and in our previous work, The main variation in method is to use a cheaper monomer,

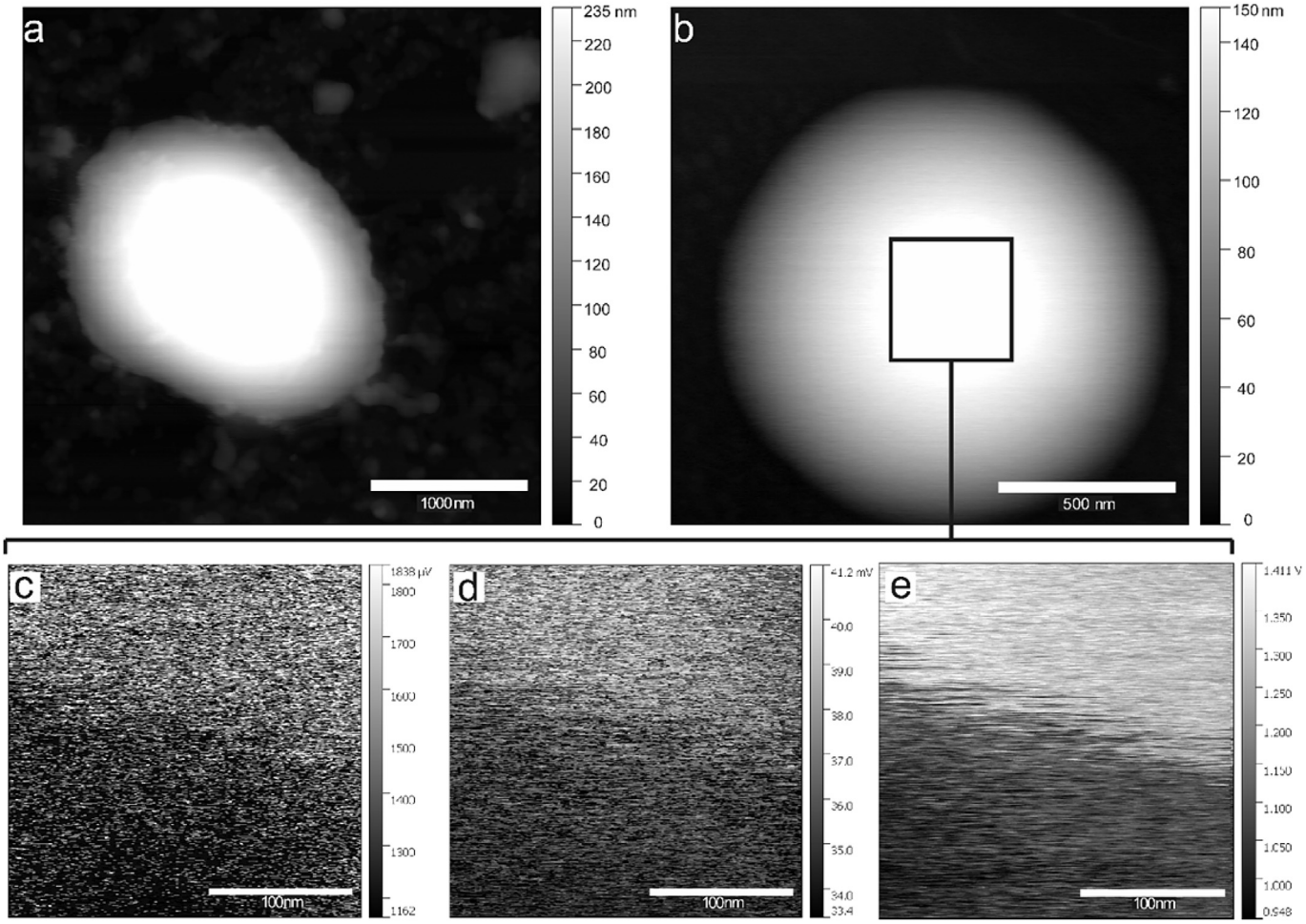


Fig. 6. AFM height images (a–b) and property maps (c–e) for PGA synthesized at 70 °C on hydrophilic mica (a) and hydrophobic HOPG (b–e). Contrast is observed in the property maps for adhesion (c), stiffness (d) and deformation (e) without corresponding change in height, indicating the material has segregated at the nanoscale in this region. Scale bars are a:1 μm, b:500 nm and c–e:100 nm.

either the dimethyl ester or the unesterified adipic acid, which also have a slightly improved atom efficiency. However, this requires a higher synthesis temperature, a longer reaction time and carrying out the reaction under vacuum to achieve a similar conversion. While our methodology results in extra expense in terms of monomer, there would be significant trade-offs in term of simplicity of procedure (Table 3SI). At laboratory scale synthesis the difference in cost between the monomers is relatively small and the difference would be likely to decrease in the event of scale up, so the cost advantages of using the less reactive monomers is likely to be relatively small.

From contact angle experiments, materials with different degree of branching showed different hydrophobicity with values ranging from 56° to 72°. AFM results are consistent with the amphiphilic nature of PGA, which can adhere to both hydrophilic and hydrophobic surfaces. These observations help to justify the ability of PGA to incorporate both hydrophilic and hydrophobic drugs and achieve a slow and prolonged release profile. Finally our DSC data showed T_g values ranging from −49 °C to −34 °C for the most and the least branched material respectively. Changes in branching thus have a number of effects relating to solvated volume which affect the apparent molecular weight of the polymer, the hydrophobicity of the polymer and its mobility. These properties may affect the performance of the polymer in drug delivery applications.

All these data taken together reveal a tunable branched architecture associated with highly tailored properties, underpinning the potential of PGA as a new biocompatible and biodegradable matrix in polymer-drug based technologies. However, the synthesis of linear polymers remains the most appealing application of a biological catalyst compared to a metallic or synthetic one.

4. Conclusions

We have presented a systematic investigation of Poly(glycerol adipate). PGA is composed of nontoxic biological residues and, as widely shown in literature, can be synthesized in high yield in scalable quantities. Working in the range 40–50 °C, slightly below the protein denaturation temperature and so with high activity, Novozym 435 showed a reproducible regioselectivity towards the primary alcohol relative to the secondary equal to 70: 30. Passing from 50 °C to 70 °C, an increasing tri-substitution from ~5% up to ~30% was observed. Different molecular weights of PGA at different reaction temperatures support NMR indications. In addition, a smaller observed molecular weight of branched polymers relative to calculated DP shows they have a smaller solvated volume than

the unbranched variants. It was observed that upon increasing the branching degree of PGA, the hydrophobicity of the material increased while the T_g decreases. Contact angle and AFM measurements emphasized the amphiphilic nature of PGA.

All these data taken together show a direct way of controlling the branched architecture of PGA, tuning T_g and contact angle, simply by varying synthesis temperature. Hence, we foresee PGA as a component for engineering solid dispersions or nanoparticles with interesting possible healthcare applications.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.polymer.2016.02.036.

References

[1] V. Siracusa, et al., Biodegradable polymers for food packaging: a review, Trends Food Sci. Technol. 19 (12) (2008) 634–643.
[2] A. Muñoz-Bonilla, M. Fernández-García, Polymeric materials with antimicrobial activity, Prog. Polym. Sci. 37 (2) (2012) 281–339.
[3] Y. Yang, et al., Chemoenzymatic synthesis of polymeric materials using lipases as catalysts: a review, Biotechnol. Adv. 32 (3) (2014) 642–651.
[4] A. Alla, et al., Acylated and hydroxylated polyamides derived from l-tartaric acid, Polymer 46 (9) (2005) 2854–2861.
[5] Y. Yu, et al., Lipase/esterase-catalyzed synthesis of aliphatic polyesters via polycondensation: a review, Process Biochem. 47 (7) (2012) 1027–1036.
[6] A.M. Klibanov, Improving enzymes by using them in organic solvents, Nature 409 (6817) (2001) 241–246.
[7] I.K. Varma, et al., Enzyme catalyzed synthesis of polyesters, Prog. Polym. Sci. 30 (10) (2005) 949–981.
[8] A. Idris, A. Bukhari, Immobilized candida antarctica lipase B: hydration, stripping off and application in ring opening polyester synthesis, Biotechnol. Adv. 30 (3) (2012) 550–563.
[9] M.J. Veld, A.A. Palmans, Hydrolases part I: enzyme mechanism, selectivity and control in the synthesis of well-defined polymers, in: A.R.A. Palmans, A. Heise (Eds.), Enzymatic Polymerisation, Springer Berlin Heidelberg, 2011, pp. 55–78.
[10] J.-i. Kadokawa, S. Kobayashi, Polymer synthesis by enzymatic catalysis, Curr. Opin. Chem. Biol. 14 (2) (2010) 145–153.
[11] J. Zhang, et al., Recent developments in lipase-catalyzed synthesis of polymeric materials, Process Biochem. 49 (5) (2014) 797–806.
[12] R.A. Gross, M. Ganesh, W. Lu, Enzyme-catalysis breathes new life into polyester condensation polymerizations, Trends Biotechnol. 28 (8) (2010) 435–443.
[13] S. Kobayashi, Lipase-catalyzed polyester synthesis – a green polymer chemistry, Proc. Jpn. Acad. Ser. B 86 (4) (2010) 338–365.
[14] S. Kobayashi, A. Makino, Enzymatic polymer synthesis: an opportunity for green polymer chemistry, Chem. Rev. 109 (11) (2009) 5288–5353.
[15] A.-C. Albertsson, R.K. Srivastava, Recent developments in enzyme-catalyzed ring-opening polymerization, Adv. Drug Deliv. Rev. 60 (9) (2008) 1077–1093.
[16] S. Kobayashi, Recent developments in lipase-catalyzed synthesis of polyesters, Macromol. Rapid Commun. 30 (4–5) (2009) 237–266.
[17] S. Hari Krishna, N.G. Karanth, Lipases and lipase-catalyzed esterification reactions in nonaqueous media, Catal. Rev. 44 (4) (2002) 499–591.
[18] Z. Yang, et al., Enzymatic synthesis of poly(butylene-co-sebacate-co-glycolate) copolyesters and evaluation of the copolymer nanoparticles as biodegradable carriers for doxorubicin delivery, Macromolecules 46 (5) (2013) 1743–1753.
[19] L. Iglesias, et al., A simple enzymatic procedure for the synthesis of a hydroxylated polyester from glycerol and adipic acid, Biotechnol. Tech. 13 (12) (1999) 923–926.
[20] N. Miletić, A. Nastasović, K. Loos, Immobilization of biocatalysts for enzymatic polymerizations: possibilities, advantages, applications, Bioresour. Technol. 115 (0) (2012) 126–135.
[21] B.J. Kline, E.J. Beckman, A.J. Russell, One-step biocatalytic synthesis of linear polyesters with pendant hydroxyl groups, J. Am. Chem. Soc. 120 (37) (1998) 9475–9480.
[22] C. Korupp, et al., Scaleup of lipase-catalyzed polyester synthesis, Org. Process Res. Dev. 14 (5) (2010) 1118–1124.
[23] C. Scholz, J. Kressler (Eds.), Tailored Polymer Architectures for Pharmaceutical and Biomedical Applications, ACS Symposium Series, vol. 1135, American Chemical Society, 2013, 0.
[24] H. Uyama, K. Inada, S. Kobayashi, Regioselectivity control in lipase-catalyzed polymerization of divinyl sebacate and triols, Macromol. Biosci. 1 (1) (2001) 40–44.
[25] Y. Yang, et al., Poly(oleic diacid-co-glycerol): comparison of polymer structure resulting from chemical and lipase catalysis, Macromolecules 44 (7) (2011) 1977–1985.
[26] P. Kallinteri, et al., Novel functionalized biodegradable polymers for nanoparticle drug delivery systems, Biomacromolecules 6 (4) (2005) 1885–1894.
[27] S. Puri, et al., Drug incorporation and release of water soluble drugs from novel functionalised poly(glycerol adipate) nanoparticles, J. Control. Release 125 (1) (2008) 59–67.
[28] V.M. Weiss, et al., Formation of structured polygonal nanoparticles by phase-separated comb-like polymers, Macromol. Rapid Commun. 33 (1) (2012) 35–40.
[29] M. de Geus, et al., Performance polymers from renewable monomers: high molecular weight poly(pentadecalactone) for fiber applications, Polym. Chem. 1 (4) (2010) 525–533.
[30] V.T. Wyatt, G.D. Strahan, Degree of branching in hyperbranched poly(glycerol-co-diacid)s synthesized in toluene, Polymers 4 (1) (2012) 396–407.
[31] T. Naolou, The behavior of fatty acid modified poly(glycerol adipate) at the air/water interface, Colloids Surf. A Physicochem. Eng. Asp. 468 (2015) 22–30.
[32] K.A. Distel, G. Zhu, P. Wang, Biocatalysis using an organic-soluble enzyme for the preparation of poly(lactic acid) in organic solvents, Bioresour. Technol. 96 (5) (2005) 617–623.
[33] W.H. Carothers, Polymers and polyfunctionality, Trans. Faraday Soc. 32 (0) (1936) 39–49.
[34] M. Gaborieau, P. Castignolles, Size-exclusion chromatography (SEC) of branched polymers and polysaccharides, Anal. Bioanal. Chem. 399 (4) (2011) 1413–1423.
[35] D. Pfefferkorn, et al., Crystallization and melting of poly(glycerol adipate)-based graft copolymers with single and double crystallizable side chains, J. Polym. Sci. Part B Polym. Phys. 51 (21) (2013) 1581–1591.
[36] A.S. Kulshrestha, W. Gao, R.A. Gross, Glycerol copolyesters: control of branching and molecular weight using a lipase catalyst, Macromolecules 38 (8) (2005) 3193–3204.
[37] I. Francolini, et al., Antifouling polyurethanes to fight device-related staphylococcal infections: synthesis, characterization, and antibiofilm efficacy, Pathogens Dis. 70 (3) (2014) 401–407.
[38] X. Luo, et al., The relationship between the degree of branching and glass transition temperature of branched polyethylene: experiment and simulation, Polym. Chem. 5 (4) (2014) 1305–1312.