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Adharis, Azis; Loos, Katja

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Green Synthesis of Glycopolymers Using an Enzymatic Approach

*Azis Adharis and Katja Loos**

Dedicated to the memory of Prof. Reimund Stadler, who, although no longer with us, continues to inspire the field

β-Glucosidase and horseradish peroxidase (HRP) are used as biocatalysts in aqueous solution for the enzymatic synthesis of glycomonomers and the respective enzymatic polymerization toward glycopolymers. The biocatalytically synthesized monomers contain (meth)acrylate functionalities that are able to be polymerized by an enzyme-initiated polymerization using an HRP/hydrogen peroxide/acetylacetone ternary system. The structure of the glycomonomers and the respective glycopolymers as well as the monomer conversion after the reaction are determined by ^{1}H NMR spectroscopy. The synthesized glycopolymers have a dispersity and a number-average molecular weight up to 5.8 and 297 kg mol^{-1}, respectively. Thermal and degradation properties of the glycopolymers are studied by differential scanning calorimetry and thermogravimetric analysis. In addition, preparation of glycopolymers via conventional free radical polymerization is performed and the properties of the obtained polymers are compared with the enzymatically synthesized glycopolymers.

1. Introduction

Glycopolymers are defined as synthetic polymers having pendant saccharide groups such as monosaccharides, disaccharides, oligosaccharides, or combinations thereof.[1] Glycopolymers are well known to be able to mimic glycolipids and glycoproteins, the macromolecules mainly involved in cell interactions with sugar-binding proteins, for example, in intercellular recognition, cell-cell adhesion, and cell differentiation. As a result, glycopolymers have been studied for various applications including gene therapy, drug delivery, disease inhibition, and biosensors.[2–6] The group of Reimund Stadler was working extensively on glyco-hybrid structures such as carbohydrate/polysaccharide modified polysiloxane brush systems,[7–11] polystyrene rod-coil systems,[12,13] and modified surfaces.[14] This work was extended in recent years to continue the efforts of the Stadler group.[15–29]

The synthesis of glycopolymers was summarized comprehensively in recent years—showing a wide range of possible synthetic methods with the majority of reports applying chain-growth polymerization mechanism either in controlled or uncontrolled fashion.[30–32] To the best of our knowledge, none of these reports utilized enzymes as alternative catalysts to synthesize glycopolymers. The role of enzymes was so far limited to the preparation of sugar-based monomers to avoid tedious protection steps of the saccharide-hydroxyl groups in conventional synthetic reactions.[33–40] In an effort to achieve complete green and sustainable processes in glycopolymer synthesis, enzymes are ideal candidates as a catalyst for the polymerization since they are nontoxic, obtained from renewable materials, and typically work under mild reaction conditions.[41–47]

Horseradish peroxidase (HRP) is one of the oxidoreductase enzymes that have been widely reported in mediating enzymatic polymerization of vinyl monomers[48–50] and the polymerization of phenol and aniline derivatives via oxidative couplings.[51] The active site of HRP contains an iron-porphyrin complex to generate free radicals in the presence of hydrogen peroxide substrates. While the versatility of HRP was demonstrated with different polymerizable groups, the polymerization of vinyl monomers derived from natural resources is rarely reported. For example, Singh and Kaplan studied HRP-mediated free radical polymerization (FRP) of the enzymatically synthesized ascorbate-based methacrylate/styrene monomers.[52,53] However, toxic trifluoroethanol was generated during the monomer synthesis which provides a disadvantage of this system in terms of eco-friendliness.

In this report, we present an HRP-mediated synthesis of glycopolymers at room temperature in aqueous solution. The glycopolymers consist of poly(2-(β-glucosyloxy)ethyl acrylate) (PGEA), poly(2-(β-glucosyloxy)ethyl methacrylate) (PGEMA), and poly(4-(β-glucosyloxy)butyl acrylate) (PGBA). The used glycomonomers (GEA, GEMA, and GBA) were synthesized by β-glucosidase in the thermodynamically controlled reverse hydrolysis reactions as previously reported by us.[40] Hence, the synthesis of the reported glycopolymers is achieved through a fully enzymatic pathway in the course of preparation of both the monomers as

Dr. A. Adharis, Prof. K. Loos
Macromolecular Chemistry and New Polymeric Materials
Zernike Institute for Advanced Materials
University of Groningen
Nijenborgh 4, 9747 AG Groningen, The Netherlands
E-mail: k.u.loos@rug.nl

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well as the polymers. Additionally, the same glycopolymers were synthesized by conventional FRP in order to compare the properties of the polymers prepared by two different methods. All reported glycopolymers were successfully characterized by ^{1}H and ^{13}C NMR spectroscopy, size exclusion chromatography, differential scanning calorimetry, and thermogravimetric analysis.

2. Experimental Section

2.1. Materials

2-Hydroxyethyl acrylate (HEA) 96%, 2-hydroxyethyl methacrylate (HEMA) 97%, β-glucosidase from almonds (activity ≥2 units mg^{-1} solid), and peroxidase from HRP type I (MW ≈ 44 kDa) with an activity of 88 pyrogallol units mg^{-1} solid were purchased from Sigma-Aldrich. One pyrogallol unit was defined as the amount of enzyme that converts pyrogallol to 1.0 mg purpurogallin in 20 s (pH: 6.0, temperature: 20 °C). 4-Hydroxybutyl acrylate (HBA) 95% was acquired from ABCR Chemicals. Acetylacetone (ACAC) 99+%, α-D(+)-glucose ≥99%, potassium persulfate (KPS) 99+%, 1,4-dioxane, and hydrogen peroxide (H_2O_2) 35 wt% solution in water was obtained from Acros Organics. Acetone, chloroform ($CHCl_3$), and methanol (MeOH) were acquired from Biosolve BV. All chemicals were used as received.

2.2. Characterization

2.2.1. Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H NMR and ^{13}C NMR spectra were measured on a 400 and 300 MHz Varian VXR Spectrometer, respectively, using deuterium oxide (99.9 atom % D, Sigma-Aldrich) as the solvent. The acquired spectra were processed by MestReNova Software from Mestrelab Research S.L.

2.2.2. Size Exclusion Chromatography (SEC)

SEC was done on a Viscotek GPCmax equipped with model 302 TDA detectors and DMF containing 0.01 M LiBr was used as the eluent at a flow rate of 1.0 mL min^{-1}. The equipment was accompanied by a guard column (PSS-GRAM, 10 μm, 5 cm) and two analytical columns (PSS-GRAM-1000/30 Å, 10 μm, 30 cm). The temperature for the columns and detectors were at 50 °C. The polymeric samples were filtered through a 0.45 μm PTFE filter prior to injection. Narrow PMMA standards were utilized for calibration and molecular weights were calculated by the universal calibration method using the refractive index increment of PMMA (0.063 mL g^{-1}).

2.2.3. Differential Scanning Calorimetry (DSC)

DSC measurements were executed on a DSC Q1000 from TA Instruments by heating the samples to 200 °C. The heating and cooling rates were maintained at 10 °C min^{-1}.

2.2.4. Thermogravimetric Analysis (TGA)

TGA measurements were performed on a TGA 5500 from TA Instruments by heating the samples to 700 °C with the scan rate of 10 °C min^{-1} under nitrogen atmosphere. The samples were first heated up to 100 °C for 15 min to remove the adsorbed water and acetone prior to measurement. The results were analyzed using TRIOS software (v4.1) from TA Instruments.

2.3. Enzymatic Synthesis of Glycomonomers

The glycomonomers consisting of GEA, GEMA, and GBA were synthesized as reported previously.[40] For example, in a 25 mL round-bottom flask, D-glucose (0.70 g, 4 mmol) was dissolved in Milli-Q water (1.4 mL). Subsequently, HEA (12 mL), HEMA (12 mL), or HBA (12 mL) was added into the glucose solution followed by addition of dioxane (1 mL). The reaction was started by adding β-glucosidase solution (70 mg in 0.6 mL Milli-Q water). The flask was then put in water bath at 50 °C for 24 h. The synthesized glycomonomers were identified by thin layer chromatography (TLC) at retardation factor of 0.55 (GEA), 0.59 (GEMA), and 0.69 (GBA) using the eluent of $CHCl_3$/MeOH (6/1). The products were isolated by column chromatography using the same eluent as TLC while silica gel served as the stationary phase. The solvent of the collected fraction containing the products was evaporated by rotary evaporation (<40 °C) until transparent syrup is obtained.

2.4. Enzymatic Polymerization Assisted by an HRP/H_2O_2/ACAC System

In a 10 mL round-bottom flask, GEA (0.41 g, 1.49 mmol), GEMA (0.44 g, 1.49 mmol), or GBA (0.48 g, 1.49 mmol) was dissolved in phosphate buffer pH 6.0 (20 mM, 2.0 mL). The flask was then sealed with a rubber septum and purged by nitrogen for at least one hour. Subsequently, ACAC (3 μL, 29.7 μmol) and H_2O_2 (3.5 wt%, 14.4 μL, 14.9 μmol) were added into the monomer solution. The reaction was started by adding HRP from a stock solution (15.5 mg mL^{-1}, 108.5 μL, 0.038 μmol) and the flask was placed in a water bath at 25 °C. After 1 h, the reaction mixture was exposed to oxygen and the flask was immediately put in liquid nitrogen to stop the reaction. An aliquot solution (100 μL) was drawn to determine the monomer conversion by ^{1}H NMR. The synthesized polymers were isolated by precipitation of the reaction mixture in a tenfold excess of acetone and reprecipitation of the product twice. The gel-like precipitates were dried in a vacuum oven (40 °C) overnight.

In addition, control/blank reactions were performed under the same conditions as the main reaction with GEA serving as the monomer and without either HRP, H_2O_2, or ACAC in the reaction mixture. 6 h of reaction time was applied instead of one hour. No characteristic polymer peaks were observed in the ^{1}H NMR spectra of the control reactions.

2.5. FRP with KPS as Initiator

In a 10 mL round-bottom flask, GEA (0.41 g, 1.49 mmol), GEMA (0.44 g, 1.49 mmol), or GBA (0.48 g, 1.49 mmol) was dissolved in phosphate buffer pH 6.0 (20 mM, 2.0 mL). The flask was then sealed with a rubber septum and purged by nitrogen for at least one hour. The reaction was started by adding a calculated amount of KPS from a stock solution (40.14 mg mL^{-1}, 100 µL, 14.9 µmol) into the reaction mixture. Subsequently, the flask was placed in an oil bath at 50 °C. After 1 h, the reaction mixture was exposed to oxygen and the flask was immediately put in liquid nitrogen to stop the reaction. An aliquot solution (100 µL) was drawn to determine the monomer conversion by ^{1}H NMR. The synthesized polymers were isolated by precipitation of the reaction mixture in a tenfold excess of acetone and two times reprecipitation. The gel-like precipitates were dried in a vacuum oven (40 °C) overnight.

In addition, GEA was used in control/blank reactions that are conducted in the following way: first, KPS was absent from the reaction mixture and the reaction was performed at 50 °C. Second, KPS was present, but the reaction was done at 25 °C. 6 h of reaction time was applied instead of 1 h for both reactions. No characteristic polymer peaks were observed in the ^{1}H NMR spectra of the control reactions.

2.5.1. P(GEA)

Monomer conversion: 94% (enzymatic), 95% (FRP). Yield: 48% (enzymatic), 56% (FRP). ^{1}H NMR (400 MHz, D_2O, δ): 4.51 (H1-axial, J = 7.6 Hz), 3.28–4.43 (H2, H3, H4, H5, H6, H7, H8), 2.31–2.62 (H9), 1.51–2.16 (H10).^{13}C NMR (300 MHz, D_2O, δ): 176.3 (C11), 102.4 (C1β), 75.7 (C5), 75.5 (C3), 73 (C2), 69.5(C4), 67.4 (C8), 64.3 (C7), 60.7 (C6), 41.6 (C9), 34.4 (C10).

2.5.2. P(GEMA)

Monomer conversion: 56% (enzymatic), 61% (FRP). Yield: 33% (enzymatic), 35% (FRP). ^{1}H NMR (400 MHz, D_2O, δ): 4.53 (H1-axial, J = 6.8 Hz), 3.19–4.42 (H2, H3, H4, H5, H6, H7, H8), 1.57–2.26 (H10), 0.53–1.52 (-CH_3).^{13}C NMR (300 MHz, D_2O, δ): 179.6 (C11), 102.4 (C1β), 75.9 (C5), 75.7 (C3), 73 (C2), 70 (C4), 67.2 (C8), 64.8 (C7), 60.9 (C6), 44.7 (C9), 35 (C10), 17 (-CH_3).

2.5.3. P(GBA)

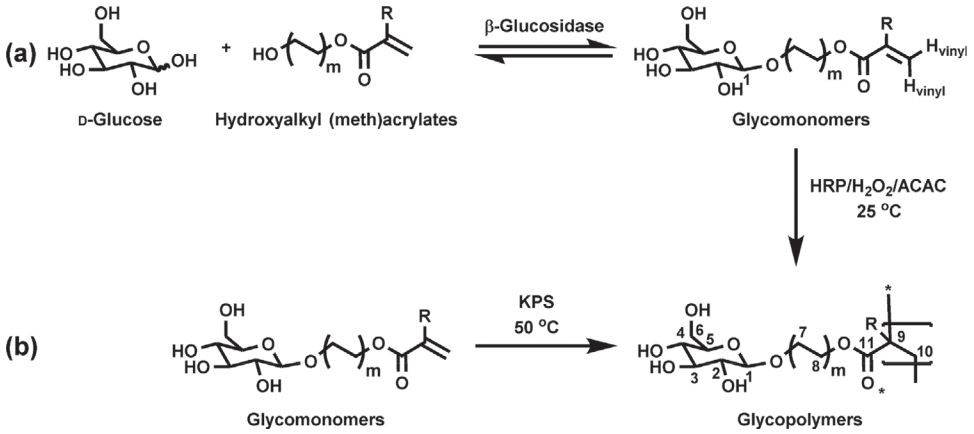
Monomer conversion: 96% (enzymatic), 97% (FRP). Yield: 55% (enzymatic), 60% (FRP). ^{1}H NMR (400 MHz, D_2O, δ): 4.46 (H1-axial, J = 7.6 Hz), 3.24–4.29 (H2, H3, H4, H5, H6, H7, H8), 2.25–2.48 (H9), 1.52–2.03 (H10, H7',H8').^{13}C NMR (300 MHz, D_2O, δ): 176.2 (C11), 102.2 (C1β), 75.9 (C5), 75.7 (C3), 73 (C2), 70 (C4), 67.6 (C7), 65.4 (C8), 60.8 (C6), 41.7 (C9), 35 (C10), 25.5 (C7'), 24.6 (C8').

3. Results and Discussion

3.1. Enzymatic Synthesis of (β-glucosyloxy)alkyl (meth)acrylates

The enzymatic synthesis of (β-glucosyloxy)alkyl (meth)acrylates was successfully performed via a biocatalytic pathway as displayed in **Scheme 1**a. Various types of glucose-based monomers were synthesized using β-glucosidase as the biocatalyst in both thermodynamically- and kinetically controlled reactions.[35,40] The former reaction is based on a reverse hydrolysis reaction of glucose with hydroxyalkyl (meth)acrylates in the equilibrium state. On the other hand, the kinetically controlled reaction uses cellobiose as glucosyl donor and hydroxyalkyl (meth)acrylates as glucosyl acceptors in the transglycosylation reaction. It was found that the thermodynamically controlled enzymatic reactions generated a better yield, used cheaper starting materials, and had fewer side products than the transglycosylation reaction.

The ^{1}H NMR spectra of GEA, GEMA, and GBA in **Figure 1**a clearly prove the successful synthesis of the monomers. For instance, the typical anomeric proton (H1) of the glucosyl unit at the axial position can be observed at 4.4 ppm. This proton indicates that anomerically pure monomers were obtained. In addition, the vinyl protons (H_{vinyl}) of the (meth)acrylate groups can be seen at 5.9–7.3 ppm. The value of peak integration for both protons is equal showing that the enzymatic synthesis is



Scheme 1. a) Enzymatically synthesized glycomonomers and glycopolymers. The monomers are composed of GEA (m = 1, R = H), GBA (m = 2, R = H), and GEMA (m = 1, R = CH_3). b) Glycopolymers prepared by conventional FRP.

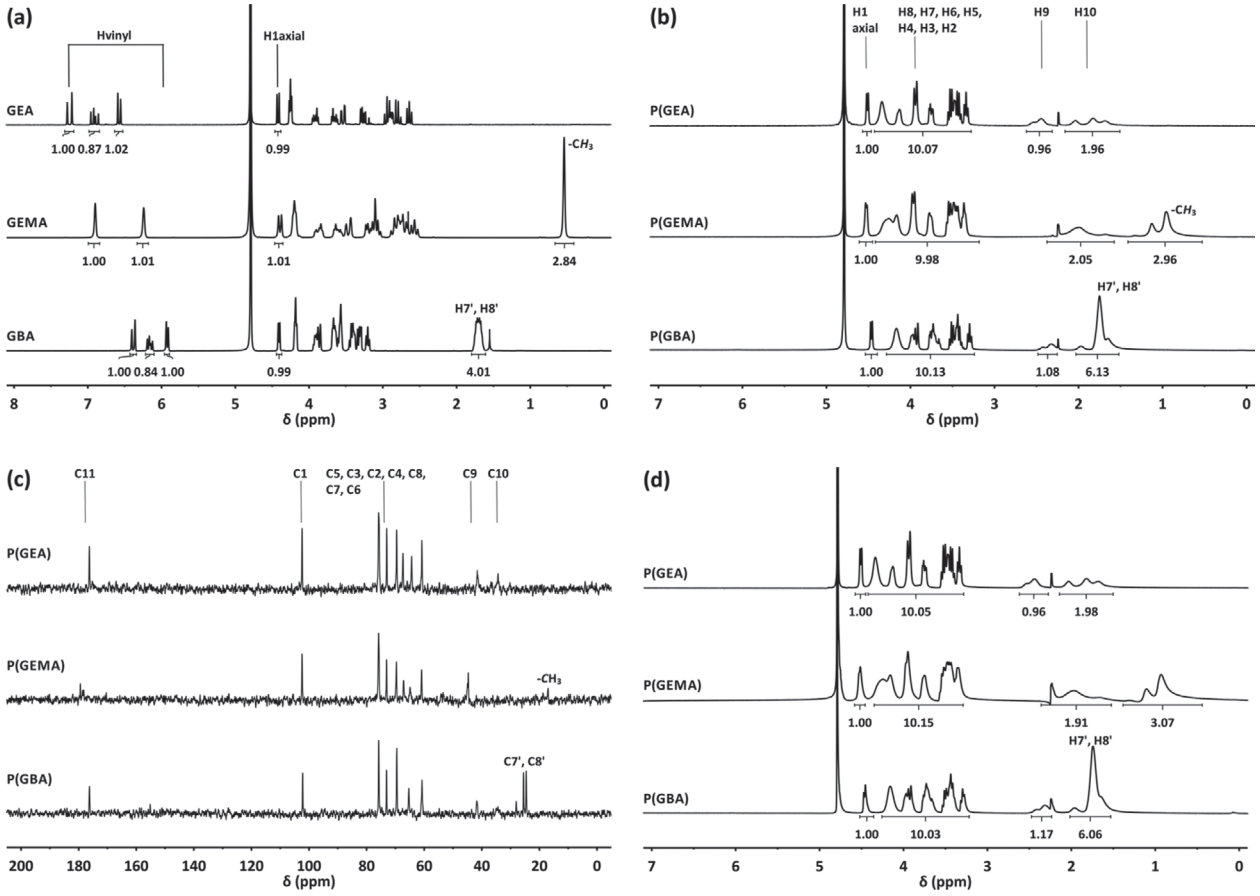


Figure 1. a) ^{1}H NMR spectra (in D_2O) of the enzymatically synthesized glycomonomers. b) ^{1}H and c) ^{13}C NMR spectra of the glycopolymers prepared by an HRP/H_2O_2/ACAC system. d) ^{1}H NMR spectra of the glycopolymers prepared by conventional FRP.

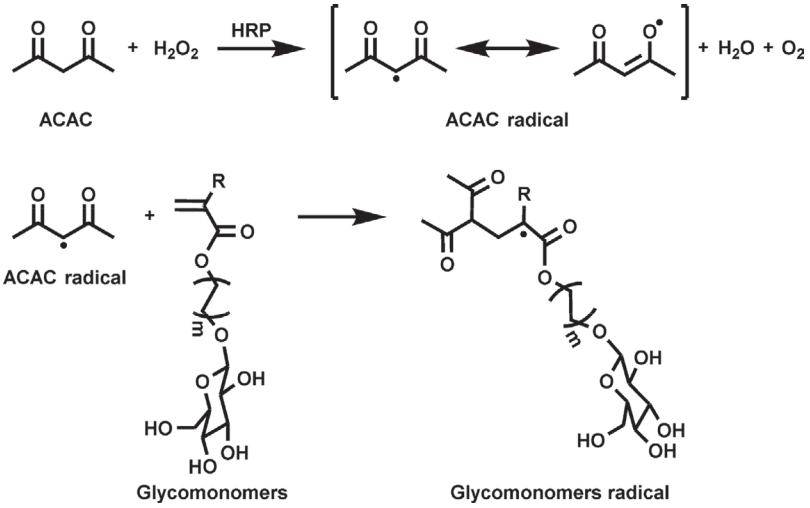
able to produce monofunctional products, which are readily polymerizable by a polyaddition mechanism. In this study, the polymerization was performed through a free radical technique either mediated by an enzyme or a chemical initiator.

3.2. Polymerization of (β-glucosyloxy)alkyl (meth)acrylates

FRP is a very robust method and the most frequently used technique for the preparation of polymers. Scheme 1a shows the aqueous FRP of the prepared monomers catalyzed by HRP at 25 °C and 1 h of reaction time. In principle, the mechanism of the HRP-mediated FRP follows the common steps as in a conventional FRP involving initiation, propagation, and termination. The polymerization requires three crucial compounds (HRP, H_2O_2, and ACAC) in order to generate a radical via a reduction-oxidation reaction (**Scheme 2**). In the performed blank reactions, in which one of these compounds was absent from the reaction mixture, no polymer was formed even after 6 h of reaction time (**Table 1**). This result shows the importance of the HRP/H_2O_2/ACAC ternary system for the creation of ACAC radicals to initiate the polymerization. Since HRP only involved in the generation of an active species, which is independent of the monomer structure, it is expected that this system is able to mediate the same reaction for other polymerizable vinyl groups. Other β-diketone molecules may be used but Maréchal and coworkers showed the excellent role of ACAC, that is able to produce the highest yield, the highest molecular weight, and the lowest dispersity of polyacrylamides, as compared to other molecules.[54]

The structure of the monomer clearly affects the conversion, that is, the acrylate-based monomers polymerize faster that the methacrylate monomer (Table 1). For example, GEA was converted to 94% after 1 h of reaction time while the GEMA conversion was only 56%. This is reasonable since acrylates create a secondary radical as the propagating end group while methacrylates form a tertiary radical which is more stable than the secondary radical. As a result, the acrylate group exhibits a higher reactivity, and thus a shorter reaction time than the methacrylate group. This is supported by a report of Buback and coworkers who observed an eighteen times higher propagation rate coefficient and a two times higher termination rate coefficient for acrylic acid compared to methacrylic acid in an aqueous FRP.[55]

Structural analysis of the synthesized glycopolymers was performed by ^{1}H NMR spectroscopy (Figure 1b). In comparison with the spectra of the monomers in Figure 1a, broad proton peaks at around 1.5–2.5 ppm were detected and can be assigned to the protons of the polymer backbone. In addition, the anomeric proton peak of the glucosyl units remained



Scheme 2. Initiation mechanism of HRP-mediated FRP of glycomonomers.

noticeable while the vinyl proton peaks of the monomer disappeared. In agreement with the ^{1}H NMR spectra, ^{13}C NMR spectra of the synthesized glycopolymers (Figure 1c) clearly show the carbon peaks of the polymer backbone at 35 and 42 ppm. These results prove the successful polymerization of the prepared glycomonomers with an intact anomeric configuration of the glucosyl units after the reaction.

For comparison purposes, we synthesized the glycopolymers using KPS as the chemical initiator at 50 °C and 1 h of reaction time (Scheme 1b). Similar ^{1}H NMR spectra of the glycopolymers were obtained for both enzymatic and conventional FRP (Figure 1d) supporting an identical structure of both glycopolymers. Interestingly, while the enzyme-mediated FRP was successfully conducted at 25 °C, the conventional FRP failed to produce the polymers at this temperature, although the initiator was present and a reaction time of 6 h was applied (see Table 1). This shows the significant advantage of using enzymatic polymerizations, which are able to catalyze the polymerization in shorter reaction times, requiring less energy and therefore reducing the cost.

The number-average molecular weights (M_n) of the synthesized glycopolymers were determined by SEC and are shown in Table 1. Even though the enzyme-mediated FRP was carried out at a lower temperature than the conventional FRP, the M_n of the glycopolymers prepared by both methods are similar. When the enzymatic polymerization was stopped at the desired time, gelation was not observed indicating the monomer still had good mobility during the reaction at room temperature.

3.3. Thermal and Degradation Properties of the Synthesized Glycopolymers

Most potential applications for glycopolymers are based on polymer solutions. Nevertheless, their properties in bulk are important as well because glycopolymers can also be used as films,[56,57] fibers,[58–60] and matrices[61,62] requiring good structural stability of the polymers, for instance against mechanical and thermal treatments.

Figure 2a shows thermograms of the enzyme-mediated glycopolymers measured by DSC. Similar results were found for the glycopolymers prepared by conventional FRP. The observed glass transition temperatures (T_gs) are summarized in **Table 2**. Considering the relatively high M_n of the synthesized glycopolymers, the T_g should not be influenced by the synthetic method used as predicted by the Flory–Fox equation.[63,64] The T_g of P(GEA) was 100 °C, which is higher than the T_g of P(GBA) with 71 °C. This can be explained by a higher free volume of P(GBA) caused by longer alkyl side chains. Moreover, the T_g of P(GEMA) was higher than the T_g of P(GEA) since the methyl group at the backbone of P(GEMA) restricts the mobility of the polymer chain. As a result, P(GEMA) requires higher temperatures than P(GEA) for the transition from the glassy state to the rubbery state of the amorphous phase of the materials.

Table 1. Overview of the synthesized glycopolymers by HRP/H_2O_2/ACAC ternary systems.

Polymer	[M]$^{a)}$:[H_2O_2]:[ACAC]:[HRP]	[M]$^{a)}$:[KPS]	$T_R$$^{b)}$	$t_R$$^{c)}$	Conv. [%]$^{d)}$	$M_{n,\,SEC}$$^{e)}$	$Đ$
P(GEA)	$100:1:2:2.58\times10^{-3}$	–	25	1	94	200	4.3
	–	100:1	50	1	95	223	5.2
P(GEMA)	$100:1:2:2.58\times10^{-3}$	–	25	1	56	190	3.8
	–	100:1	50	1	61	205	4.4
P(GBA)	$100:1:2:2.58\times10^{-3}$	–	25	1	96	262	4.7
	–	100:1	50	1	97	297	5.8
P(GEA)	$100:0:2:2.58\times10^{-3}$	–	25	6	0	–	–
	$100:1:0:2.58\times10^{-3}$	–	25	6	0	–	–
	100:1:2:0	–	25	6	0	–	–
	–	100:0	50	6	0	–	–
	–	100:1	25	6	0	–	–

$^{a)}$[Monomer] = 0.70 M; $^{b)}$Reaction temperature in °C; $^{c)}$Reaction time in hours; $^{d)}$Determined by ^{1}H NMR spectroscopy; $^{e)}$Molecular weights in kg mol^{-1}.

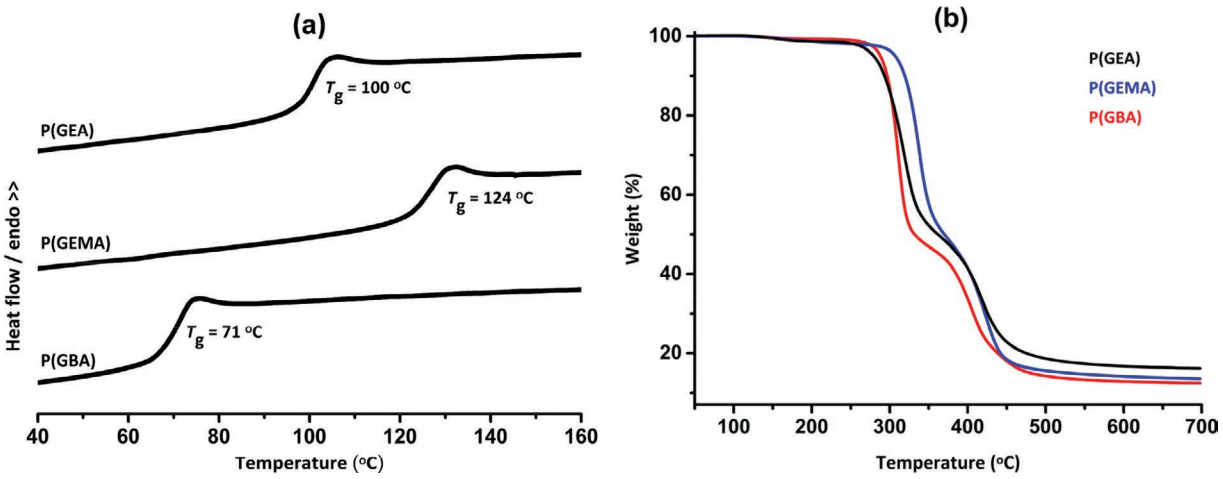


Figure 2. a) DSC thermograms recorded at 10 °C min^{-1} (second heating cycle) and b) TGA decomposition profiles of the glycopolymers synthesized by the HRP/H_2O_2/ACAC system.

Thermal stability of the enzymatically synthesized glycopolymers was examined by TGA as presented in Figure 2b. Similar results were gained for the glycopolymers synthesized by conventional FRP. The TGA profiles clearly show that the glycopolymers possess two decomposition steps under a nitrogen atmosphere. The first degradation step at around 320 °C is attributed to the decomposition of the glucosyl unit with a weight loss of about 50% while the theoretical weight loss of this unit is 53–59%. The second step at about 413 °C is related to the dissociation of the remaining polymeric chains. Moreover, the elimination of absorbed water was detected at around 150 °C since the glycopolymers are very hygroscopic. A similar observation of water elimination was reported in the literature for other types of glycopolymers.[65–67]

4. Conclusions

We have successfully synthesized glycopolymers of P(GEA), P(GEMA), and P(GBA) by FRP techniques either mediated by an enzyme or initiated by KPS. The ternary initiating system of HRP, H_2O_2, and ACAC played a pivotal role in creating the radical in the enzymatic polymerization. The acrylate-based glycomonomers were found to polymerize faster than the methacrylate monomers due to the formation of less stable radicals during the propagation reaction.

The enzymatic polymerization of glycomonomers was performed at 25 °C while the conventional reaction was done at 50 °C. Nevertheless, both glycopolymers prepared by enzymatic and chemical initiators showed a similar structure as determined by ^{1}H and ^{13}C NMR spectroscopy. In addition, these glycopolymers possess similar M_n, T_g, and T_d. The M_ns and the T_gs were in the range of 190–297 kg mol^{-1} and 71–127 °C, respectively. The synthesized glycopolymers possess two decomposition steps at around 320 °C and 413 °C based on TGA measurements.

The preparation of glycomonomers and glycopolymers were conducted in an environmentally friendly approach, a novel way towards more sustainable polymers. The enzymes used in the reactions are commercially available, thus enabling them for further development of the reactions on a large scale. However, considering the aspect of efficiency and cost of the enzymes, immobilization of the enzymes would be more beneficial in order to recover and recycle the enzymes after the reaction. In addition, the utilization of oxidoreductase in catalyzing controlled radical polymerizations start to gain much attention in recent years.[68] Therefore, future experiments will focus on biocatalytic methods for creating well-defined glycopolymer structures, which are highly interesting for biomedial applications.

Table 2. T_g and decomposition temperatures (T_d) of the synthesized glycopolymers.

Polymer	T_g [°C]	$T_{d\text{-}max1}$ [°C]	$T_{d\text{-}max2}$ [°C]
P(GEA)[a)]	100	317	417
P(GEA)[b)]	101	321	410
P(GEMA)[a)]	124	333	425
P(GEMA)[b)]	127	337	421
P(GBA)[a)]	71	309	406
P(GBA)[b)]	72	310	401

[a)]Mediated by HRP/H_2O_2/ACAC systems; [b)]Initiated by KPS.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biocatalysis, carbohydrates, glycomonomers, horseradish peroxidase, β-glucosidase

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