Preparation of water-soluble glycopolymers derived from five-membered iminosugars

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Abstract

Iminosugars are carbohydrate mimetics that constitute the main group of oligosaccharide processing enzyme inhibitors. They play an important role in cell-cell recognition processes, thereby presenting a wide range of potential therapeutic applications. In this work, we present the first examples of glycopolymers containing five-membered iminosugars. The preparation is carried out by constructing first a biocompatible water-soluble and pH-responsive 2-hydroxyethyl methacrylate (HEMA)-based copolymer by RAFT polymerization with an excellent control on the copolymer composition and low polydispersity (M_n = 19,800; M_w = 23,800; M_w/M_n = 1.2; experimental copolymer mole composition (by ^{1}H NMR): p$HEMA_{49\%}$-*co*-p$DMAEMA_{51\%}$). One-pot azidation of its primary hydroxyl groups by Mitsunobu reaction led to a full functionalization of the HEMA residues. Subsequently, a selection of alkynyl pyrrolidine iminosugars showing glycosidase inhibition properties, were anchored to the functionalized azido-polymer through CuAAC click reaction furnishing successfully the aimed water-soluble glycopolymers, which were then subjected to biological evaluation as enzyme inhibitors.

Keywords

Iminosugars, glycosidase inhibitors, pyrrolidines, CuAAC, RAFT polymerization, functionalized polymers

1. Introduction

Biological recognition processes are mainly based on carbohydrate-protein interactions [1]. These important events mediate many essential biological functions including cell-cell adhesion, cell growth, and immune defense [2–4]. In particular, glycosidases are enzymes that are involved in a large number of anabolic and catabolic biological processes necessary for cellular life such as digestion, lysosomal catabolism of glycoconjugates and glycoprotein biosynthesis [5–7]. The inhibition of the action of these enzymes can have a crucial effect on the composition and final conformation of the protein and, consequently, may alter the processes of maturation, transport and secretion, altering the cell-cell and virus-cell recognition phenomena [8]. Therefore, inhibitors of glycosidases are posited as potential drugs for the treatment of several types of diseases [9–13]. Imino-sugars [14,15], both synthetic and natural, are small molecules that contain a nitrogen atom instead of the endocyclic oxygen of carbohydrates. They constitute the most extensive group of carbohydrate mimetics acting as glycosidase inhibitors, thus presenting potential for the development of new therapeutic agents.

The binding affinity of multivalent assemblies of saccharides has been extensively studied on lectins [4,16–20]. The increase in the binding affinity and specificity has been laid to "the cluster glycoside effect" which is attributed to the enhanced number and cooperativity of possible binding events [21,22]. On their side, many glycosidases and other carbohydrate-processing enzymes are generally monomeric, and bind to a single carbohydrate mimetic with significant affinity and specificity. Even more, many glycosidases possess deep catalytic sites. For many years, the design of glycosidase inhibitors was focused on the use of compounds, such as iminosugars, that mimicked the transition state corresponding to enzymatic hydrolysis [23,24].

Nevertheless, studies during the last decade have shown that multivalency can improve the inhibitory properties of carbohydrate processing enzymes [25–27]. For the preparation of multivalent assemblies, several platforms such as macrocycles, fullerenes, calixarenes, cyclodextrins, dendrimers and nanodiamonds with the appropriate pendant iminosugar, have been used [28–31].

Recently, the study of multivalency with glycosidases and other carbohydrate processing enzymes has been extended to polymeric scaffolds with pending carbohydrate motifs, emerging as an important tool for the study of such interactions. Thus, the anchor of a bioactive compound to a polymer backbone provides the possibility to target the polymer to a particular position *in vivo*, being this feature significant due to its potential applicability in materials science and biomedicine [32,33].

Compain and co-workers [34] developed a polypeptide decorated with deoxynojirimycin (DNJ) residues, which showed an increase inhibition towards α-mannosidase. Nguyen and co-workers have recently reported [35] several glycopolymers bearing heparan sulfate disaccharides attached to a synthetic polymer backbone, as inhibitors of heparanase in the low nanomolar range. On their side, S. G. Gouin and co-workers [36] have also reported polyvalent polymeric alkynyl-dextran and azido-iminosugars with pending DNJ or deoxymannojirimicin (DMJ), depending their activity on the structure of the pendant iminosugars. Amphiphilic glycosylated polymers that are able to self-assemble have also been reported [37].

We have recently published the synthesis and biological evaluation of a small library of pyrrolidine-triazole hybrid molecules as selective inhibitors of β-glucosidase (almond) and α-galactosidase (coffee bean). The most potent inhibitors presented an aryltriazole moiety, with compounds **1-3** proving to be the most active (Figure 1) [38]. Moreover, halo-aryl derivatives of **1** and **2** showed good inhibitory activities against lysosomal β-glucocerebrosidase [39]. These findings remark that the incorporation of (hetero)aromatic residues into the iminosugar backbone improves the potency and selectivity of the iminosugars as enzyme inhibitors, as it has been shown by our research group [38,40,41] and by others [42,43].

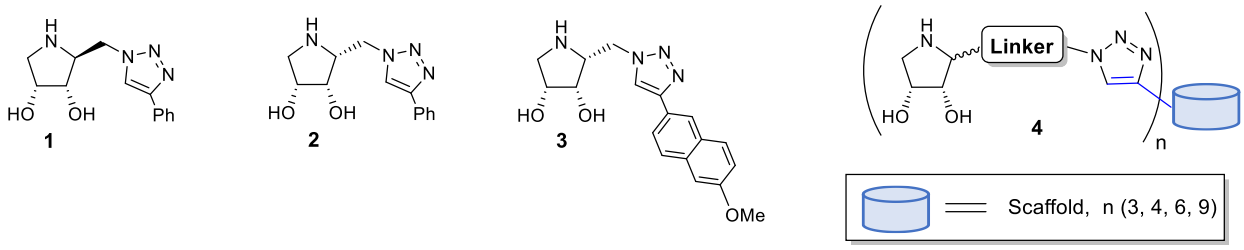
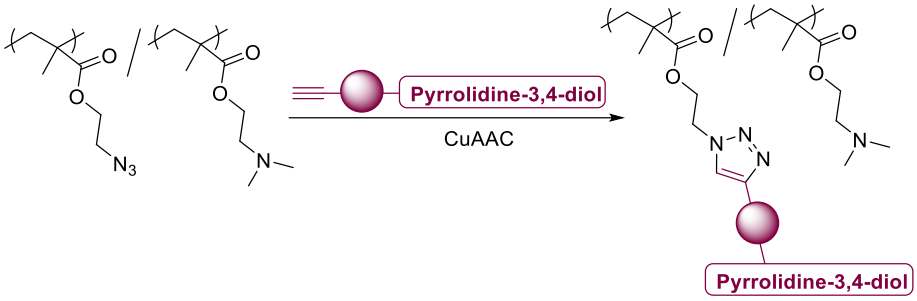


Figure 1. Pyrrolidine triazole hybrid molecules and multivalent assemblies

We have also prepared a library of multivalent pyrrolidine-derived glycoclusters **4** [44] based on the structures previously described in order to evaluate the influence of the multimeric presentation of this type of compounds in the inhibition of β-glucosidases. These multimeric structures constitute one of the very few examples of multivalent systems based on five-membered iminosugars, and the only ones with free secondary amines, i.e. avoiding attaching the pyrrolidine to the scaffold by the endocyclic nitrogen. Some of these multivalent inhibitors presented a modest multivalent effect, which may be mainly explained in terms of the statistical rebinding mechanism (an increased concentration of ligands in the proximity of the active site of the enzyme favours ligand rebinding).

With the aim of increasing the density of iminosugar ligands in the multivalent assemblies and studying its effect in the inhibitory properties against a panel of available commercial enzymes, we

now report the preparation and biological evaluation of several glycopolymers based on pyrrolidine-3,4-diol derivatives. As far as we are aware, this type of glycopolymers derived from five-membered iminosugars has been prepared for the first time, thus constituting a novel application to the field of glycopolymers. The strategy implies first, the building of specific functionalized copolymers by controlled polymerization as scaffolds, and second, the subsequent attachment of biologically active iminosugar derivatives. For this attachment we have used the Cu(I)-catalyzed azide-alkyne cycloaddition reaction (CuAAC) [45] (Scheme 1).



Scheme 1. General strategy for the preparation of iminoglycopolymers *via* CuAAC

2. Materials and Methods

2.1. General Methods

All chemicals used were purchased from Aldrich Chemical Co. 2-Hydroxyethyl methacrylate (HEMA) and *N*,*N*-dimethylaminoethyl methacrylate (DMAEMA) were passed through a basic alumina column and distilled before use. Optical rotations were measured in a 1.0 cm or 1.0 dm tube with a Jasco P-2000 spectropolarimeter. Infrared spectra were recorded with a Jasco FTIR-410 spectrophotometer and FT/IR 4200 spectrometer equipped with ATR. TLC were performed on silica gel 60 F_{254} (Merck), with detection by UV light charring with *p*-anisaldehyde, $KMnO_4$, ninhydrin or with reagent [$(NH_4)_6MoO_4$, $Ce(SO_4)_2$, H_2SO_4, H_2O]. Silica gel 60 (Merck, 40-60 and 63-200 μm) was used for preparative chromatography. Gel permeation chromatography (GPC) analyses were performed using a Waters apparatus equipped with a Waters 2414 refractive index detector and two Styragel® HR columns (7.8 x 300 mm^2) linked in series, thermostatted at 40 ºC, and using *N*,*N*-dimethylformamide (DMF) as the mobile phase at a flow rate of 0.5 mL/min. Molecular weights were estimated against methyl methacrylate standards. The thermal behavior of the polymers was examined by Differential Scanning Calorimetry (DSC), using a TA DSC Q-200 Instrument calibrated with indium. DSC data were obtained from samples of 1–5 mg at

heating/cooling rates of 10 °C min^{-1} under a nitrogen flow (flow rate 50 mL min^{-1}). The glass transition temperatures were determined at a heating rate of 10 °C min^{-1} from rapidly melt-quenched polymer samples. Thermogravimetric analyses (TGAs) were performed under a nitrogen atmosphere (flow rate 100 mL min^{-1}) with a Universal V4.3A TA Instrument at a heating rate of 10 °C min^{-1}. NMR and mass spectra were registered in CITIUS (University of Seville). ^{1}H and ^{13}C NMR spectra were recorded at 300 K with a Bruker AMX300, AMX-500 and Bruker Advance AV300, AV-500 for solutions in DMSO-d_6 and CD_3OD. Chemical shifts (δ) are reported as parts per million downfield from Me_4Si and *J* in Hz. *J* are assigned and not repeated. All the assignments were confirmed by COSY and HSQC experiments. High resolution mass spectra were recorded on a Q-Exactive spectrometer. The wettability of each sample was determined by contact angle (CA) measurements using double-distilled water and a CA meter (SEO-Phoenix 300 Touch Automatic Contact Angle Analyzer). Measurements were made three seconds after application of the droplet (10 µL) at 25 ºC.

2.2. Inhibition studies with commercial enzymes

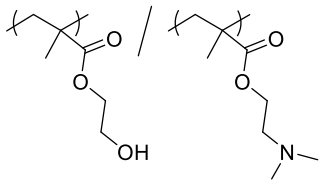
The percentage of inhibition towards the corresponding glycosidase was determined in the presence of 1.0 mM of the inhibitor on the well. Each enzymatic assay (final volume 0.12 mL) contains 0.01-0.5 units/mL of the enzyme and 4.2 mM aqueous solution of the appropriate *p*-nitrophenyl glycopyranoside (substrate) buffered to the optimal pH of the enzyme. Enzyme and inhibitor were pre-incubated for 5 min at r.t. and the reaction started by addition of the substrate. After 20 min of incubation at 37 °C, the reaction was stopped by the addition of 0.1 mL of sodium borate solution (pH 9.8). The *p*-nitrophenolate formed was measured by visible absorption spectroscopy at 405 nm. Under these conditions, the *p*-nitrophenolate released led to optical densities linear with both, reaction time and concentration of the enzyme.

2.3. Preparation of iminosugars as anchoring moieties.

Compounds **6**, **9**, **12**, **14** and **16** were prepared according to Scheme 2. The experimental procedures are included in the Supporting Information.

2.4. Preparation of functionalized polymers as chemical scaffolds

Synthesis of pHEMA$_{49\%}$-random-pDMAEMA$_{51\%}$ (Polymer A)

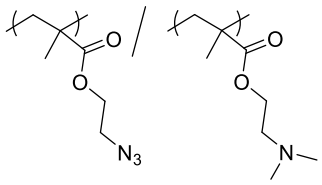


The preparation of pHEMA$_{50\%}$-*random*-pDMAEMA$_{50\%}$ (Scheme 4) was conducted by Reversible Addition−Fragmentation Chain-Transfer (RAFT) polymerization [46]. Firstly, 2-cyano-2-propyl-4-cyanobenzodithioate (CPCB) RAFT agent (42 mg, 0.173 mmol), 2-hydroxyethyl methacrylate (HEMA monomer, 2.1 mL, 17.28 mmol), and 2-(*N*,*N*-dimethylamino)ethyl methacrylate (DMAEMA monomer, 2.9 mL, 17.28 mmol) were degassed for 20 min.

Secondly, anhydrous ethanol (7.5 mL, final monomer concentration: 40 w/v %), previously purged with Argon for 30 min, and 4,4'-azobis(4-cyanopentanoic acid) (ACVA, 15 mg, 0.0525 mmol, CPCB/ACVA molar ratio = 3:1) were added, and the resulting solution was degassed for other 5 min prior to immersion in an oil bath set at 65 °C. The reaction solution was stirred for 8 hours and quenched by immersion in liquid nitrogen. Ethanol (50 mL) was added to the reaction solution, the mixture stirred under air and the polymer precipitated when the ethanolic solution was poured onto cold *tert*-butyl methyl ether (TBME, 400 mL). The final pink polymer was filtered, washed with cold TBME and dried under vacuum, leading to polymer **A** in high yield (98%). M_n = 19,800; M_w = 23,800; M_w/M_n = 1.2. Experimental mole copolymer composition (determined by ^{1}H NMR): pHEMA$_{49\%}$-*random*-pDMAEMA$_{51\%}$.

IR (ν cm^{-1}) 3354 (O-H), 2950 (C-H st), 1718 (C=O st), 1271, 1237 (C-O st ester), 1154 (C-N st amine). ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm) 4.04 (br. s, 4H, COOC$\underline{H}_2$, HEMA and DMAEMA), 3.75 (bs, 2H, COOCH$_2$C$\underline{H}_2$, HEMA), 2.58 (bs, 2H, COOCH$_2$C$\underline{H}_2$, DMAEMA), 2.35-2.17 (m, 6H, N(C$\underline{H}_3$)$_2$, DMA), 1.99-1.68 (m, 4H, -C$\underline{H}_2$-C(CH$_3$)-, DMAEMA, HEMA), 1.13-0.71 (m, 6H, -CH$_2$-C(C$\underline{H}_3$)-, DMAEMA, HEMA). ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm) 177.1 (C=O), 66.3-66.1 (COOCH$_2$C$\underline{H}_2$, DMAEMA and HEMA), 62.2 (COOC$\underline{H}_2$, HEMA and DMAEMA), 59.0 (-C$\underline{H}_2$-C(CH$_3$)-, DMAEMA and HEMA), 45.5 (N(C$\underline{H}_3$)$_2$, DMAEMA), 18.4 (-CH$_2$-C(C$\underline{H}_3$)-, DMAEMA and HEMA).

Azidation of pHEMA$_{49\%}$-pDMAEMA$_{51\%}$: preparation of pAEMA$_{49\%}$-pDMAEMA$_{51\%}$ (Polymer B)



To a solution of triphenylphosphine (8.322 g, 31.73 mmol,) in dry THF (40 mL) at 0 ºC, a commercial 40% solution of diethylazodicarboxylate (DEAD) in toluene (13.8 mL, 31.73 mmol,) was added dropwise, and the mixture was stirred for 5 min.

The sequential dropwise addition of diphenylphosphoryl azide (DPPA) (6.5 mL, 30.16 mmol,) and the solution of pHEMA$_{49\%}$-*random*-pDMAEMA$_{51\%}$ (Polymer **A**, 4.364 g, 14.86 mmol HEMA residues) in THF (45 mL) took place next. The reaction solution was stirred overnight at room temperature [47]. The mixture was added dropwise over TBME (500 mL) and the precipitated copolymer poly(2-azidoethyl methacrylate)-poly[2-(*N*,*N*-dimethylamino)ethyl methacrylate] (pAEMA-pDMAEMA, polymer **B**) was recovered by filtration. The precipitate, washed 4 times with TBME (4x125 mL) and dried under vacuum, led to a slightly colored solid (polymer **B)** with high yield (3.981 g, 84%). M_n = 19,900; M_w = 30,500; M_w/M_n = 1.5. Experimental mole copolymer composition (determined by ^{1}H NMR): pAEMA$_{49\%}$-pDMAEMA$_{51\%}$.

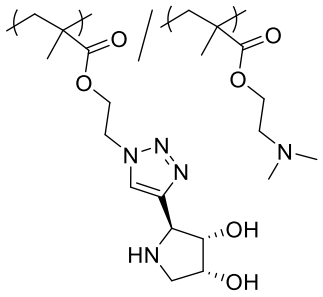
IR (ν cm^{-1}) 2952 (C-H st), 2104 (-N=N=N st), 1724 (C=O st), 1234 (C-O st ester), 1146 (C-N st amine). ^{1}H NMR (500 MHz, $CDCl_3$, δ ppm) 4.07 (bs, 4H, COOC$\underline{H}_2$, AEMA and DMAEMA), 3.47 (bs, 2H, COOCH$_2$C$\underline{H}_2$, AEMA), 2.58 (bs, 2H, COOCH$_2$C$\underline{H}_2$, DMAEMA), 2.33-2.15 (m, 6H, N(C$\underline{H}_3$)$_2$, DMA), 1.34-1.16 (m, 10H, -C$\underline{H}_2$-C(CH$_3$)-, DMAEMA, AEMA, -CH$_2$-C(C$\underline{H}_3$)-, DMAEMA, AEMA). ^{13}C NMR (125 MHz, DMSO-d_6, δ ppm) δ 178.1 (C=O), 64.9 (2C, COO$\underline{C}$H$_2$, COOCH$_2\underline{C}$H$_2$, DMAEMA), 61.3 (COO$\underline{C}$H$_2$, AEMA), 56.8 (3C, COOCH$_2\underline{C}$H$_2$, AEMA and N($\underline{C}$H$_3$)$_2$, DMAEMA), 18.5 (-CH$_2$-C($\underline{C}$H$_3$)-, DMAEMA and AEMA).

Derivatization via azide-alkyne click reaction of pAEMA$_{49\%}$-pDMAEMA$_{51\%}$: preparation of pTEMA$_{49\%}$-pDMAEMA$_{51\%}$

The general derivatization procedure [48] of the azido-based copolymer pAEMA$_{49\%}$-pDMAEMA$_{51\%}$ with selected alkynyl-iminosugars is summarized next: a degassed solution of the functionalized azide polymer (1 mmol of azide) in DMF, copper bromide (CuBr, 0.01 mmol, 1 mol%), and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 0.1 mmol, 10 mol%) were added to a two-necked, round-bottom flask. A solution of the selected alkynyl molecule in DMF (1.05 mmol, 0.7 mmol/mL) was next added over the polymer suspension. The resulting reaction mixture was degassed for 5 min prior to immersion in an oil bath set at 70 °C and heated for 24 hours. The click reaction was quenched by the addition of silicagel suspended in DMF and then filtered over a

celite/silicagel bed. The solvent was removed under vacuum to produce the derivatized polymers **C** and **D**.

Detivatization with iminosugar 6: p(TEMA-6)$_{49\%}$-pDMAEMA$_{51\%}$ (Polymer C)

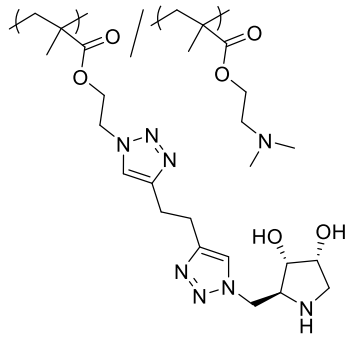


Derivatization of azide units was carried out using the procedure described above. The amounts used were: pAEMA$_{49\%}$-pDMAEMA$_{51\%}$ (300 mg, 0.982 mmol AEMA), CuBr (1.5 mg, 0.0098 mmol), DBU (15 μL, 0.098 mmol), and alkynyl derivative **6** (132 mg, 1.031 mmol) in DMF (final volume: 10 mL). The polymer was isolated as colored polymer. Yield: 215 mg (51%).

Experimental copolymer composition (determined by ^{1}H NMR): p(TEMA-6)$_{49\%}$- pDMAEMA$_{51\%}$.

IR (ν cm^{-1}) 3259 (O-H), 2925 (-C-H st), 1703 (C=O st), 1201 (C-O st ester). ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm) 8.02 (s, 1H, N-C<u>H</u>=C, triazole), 5.44-5.23 (bs, OH, pyrrolidine, TEMA-6); 4.75-4.68 (m, 2 H, COOC<u>H</u>$_2$, TEMA-6), 4.65 (bs, 2 H, COOC<u>H</u>$_2$, DMAEMA), 4.46-4.39 (m, 2H, COOCH$_2$C<u>H</u>$_2$, TEMA-6); 4.34-4.30 (m, 1H, CH$_2$C<u>H</u>OHCHOH, pyrrolidine, TEMA-6), 4.29-4.27 (m, 1H, CHOHC<u>H</u>OHCHNHtriazole, pyrrolidine, TEMA-6), 4.27-4.24 (m, 1H, CHOHC<u>H</u>NHtriazole, pyrrolidine, TEMA-6), 4.02-3.97 (br. s, 2 H, COOCH$_2$C<u>H</u>$_2$, DMAEMA), 3.53 (bs, 2H, HNC<u>H</u>$_2$CHOH, pyrrolidine, TEMA-6), 2.26-2.14 (m, 6H, N(C<u>H</u>$_3$)$_2$, DMAEMA), 1.99-1.88 (m, 6H, -CH$_2$-C(C<u>H</u>$_3$)-, TEMA-6 and DMAEMA).

Detivatization with iminosugar 12: p(TEMA-12)$_{49\%}$-pDMAEMA$_{51\%}$ (Polymer D)



Derivatization of azide units was carried out using the procedure described above. The amounts used were: pAEMA$_{49\%}$-pDMAEMA$_{51\%}$ (100 mg, 0.327 mmol AEMA), CuBr (0.5 mg, 0.00327 mmol), DBU (5 μL, 0.0327 mmol), and alkynyl derivative **12** (81 mg, 0.343 mmol) in DMF (final volume: 10 mL). The polymer was isolated as colored polymer. Yield: 86 mg (49%). Experimental copolymer composition (determined by ^{1}H NMR): p(TEMA-12)$_{49\%}$- pDMAEMA$_{51\%}$.

IR (ν cm^{-1}) 3285 (O-H), 2925 (-C-H st), 1709 (C=O st), 1205 (C-O st ester). ^{1}H NMR (500 MHz, DMSO-d_6, δ ppm) 7.83 (bs, 2H, N-C<u>H</u>=C, triazole), 5.24-5.06 (m, 2H, COOC<u>H</u>$_2$, TEMA-12), 5.04-4.92 (m, 2H, N(triazole2)C<u>H</u>$_2$CH(pyrrolidine), TEMA-12), 4.77 (bs, 2H, COOC<u>H</u>$_2$, DMAEMA), 4.62-4.51 (m, 2H, CH$_2$C<u>H</u>OHC<u>H</u>OH, pyrrolidine, TEMA-12), 4.51-4.43 (m, 2H, HNC<u>H</u>$_2$CHOH, pyrrolidine, TEMA-12), 4.33 (bs, OH, pyrrolidine, TEMA-12); 4.07-3.97 (m, 1H,

CHOHCHNHtriazole, pyrrolidine, TEMA-12), 3.94-3.79 (m, 2H, $COOCH_2CH_2$, TEMA-12), 3.59 (bs, 2 H, $COOCH_2CH_2$, DMAEMA), 2.95-2-86 (m, 4H, triazole1-CH_2CH_2-triazole2, TEMA-12), 2.83-2.79 (m, 6H, $N(CH_3)_2$, DMAEMA).

3.- Results and discussion

3.1. Preparation of iminosugar anchoring moieties as potential glycosidase inhibitors

For the preparation of the new polymers based on pyrrolidine derivatives, the CuAAC reaction between an azido functionalized copolymer pAEMA-pDMAEMA-type copolymer and an alkynyl-pyrrolidine was proposed as a general strategy (Scheme 1).

To achieve this goal, several features regarding the anchoring pyrrolidine derivatives have to be considered, i.e. (1) the spacer between the pyrrolidine-3,4-diol core and the alkyne moiety, (2) the nature (aliphatic or aromatic) and the position of the linker (N or at C-2) of the pyrrolidine moiety.

In the field of the synthesis of glycopolymers, the CuAAC reaction between azides and alkynes is of great interest because of its high efficiency and easy experimental handling [37,49]. In order to choose the most adequate pyrrolidine alkyne and get information about the spacer and the position of the linker for the subsequent preparation of the polymers *via* CuAAC, the preparation and biological evaluation of different functionalized alkynes, presenting the free or *N*-alkylated pyrrolidine nitrogen, were carried out (compounds **6, 9, 12, 14** and **16**) (Figure 2).

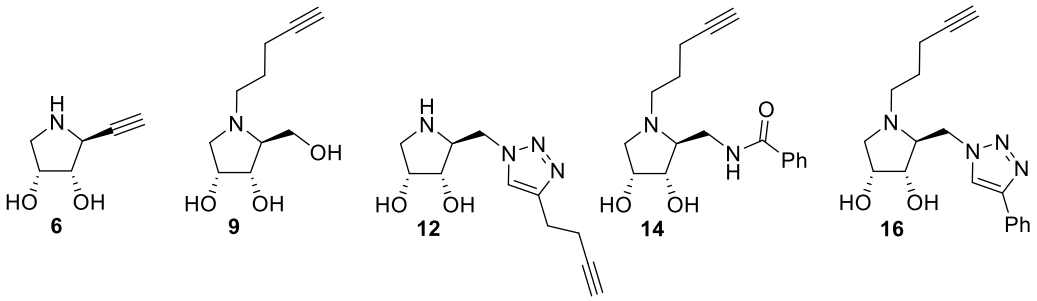
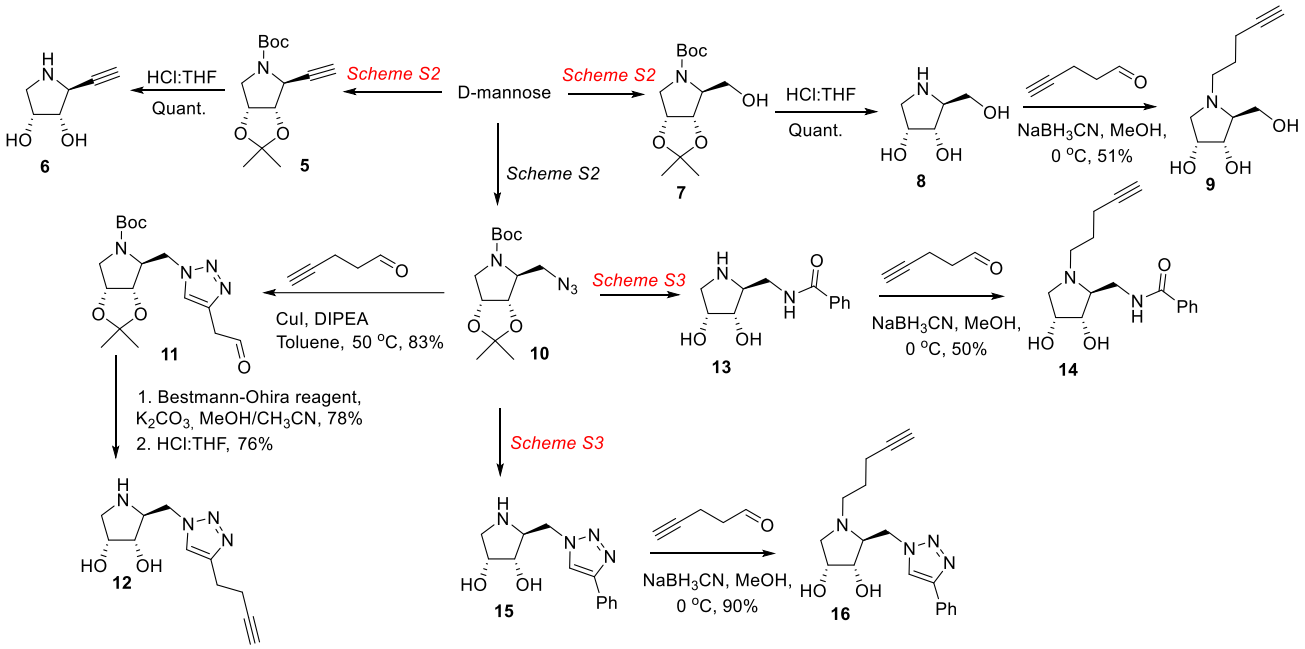


Figure 2. Alkyne derivatives for iminosugar anchoring to the polymer

Compounds **6, 9, 12, 14** and **16** were all prepared from D-mannose (Schemes S1-S4 in Supporting Information). Compound **6** was obtained after acidic deprotection of pyrrolidine alkyne **5** [38]. Deprotection of alcohol **7** gave pyrrolidine **8** in quantitative yield. Reductive amination of **8** and of the recently reported **13** and **15** [38] with pent-4-ynal [50] yielded the corresponding *N*-

alkylated trihydroxy-pyrrolidine derivative **9** and *N*-alkylated dihydroxy-pyrrolidine derivatives **14** and **16** in moderate-to good yields.

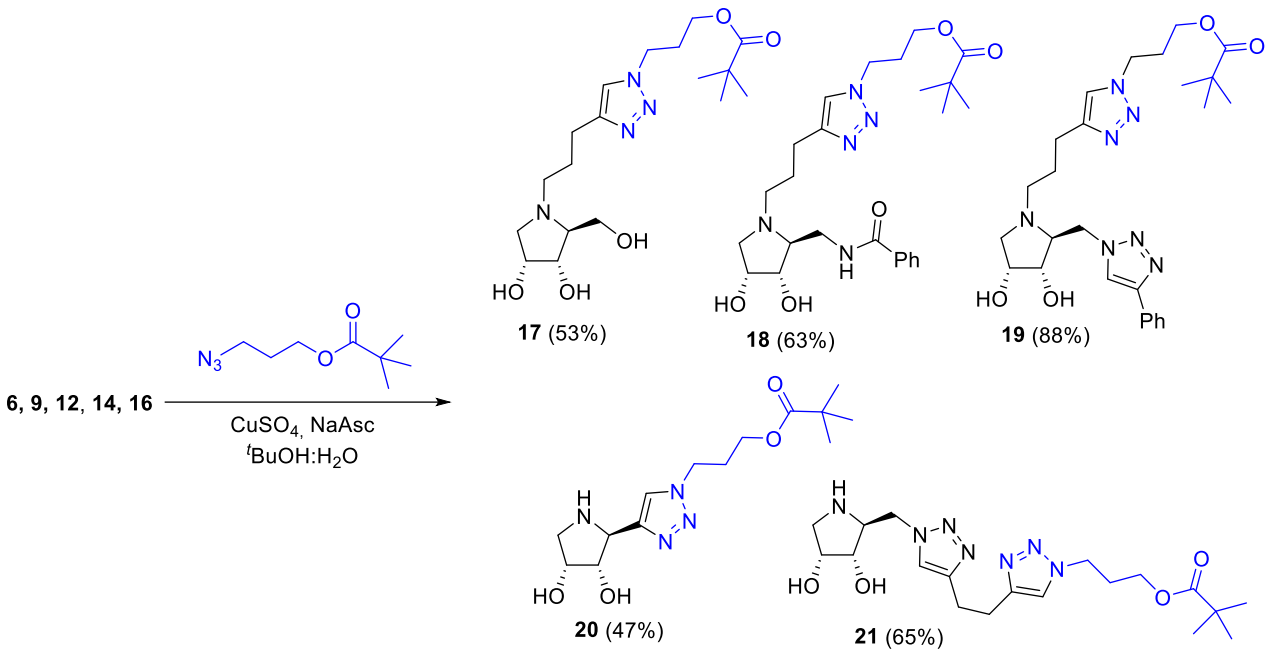
On the other hand, CuAAC reaction of protected pyrrolidine azide **10** with pent-4-ynal afforded triazole derivative **11**, which after Bestmann-Ohira reaction and subsequent acidic deprotection afforded compound **12** (Scheme 2).



Scheme 2: Preparation of iminosugars **6**, **9**, **12**, **14** and **16** from D-mannose

3.2. Glycosidase inhibition of iminosugar derivatives towards commercial enzymes

The corresponding monovalent reference compounds (**17-21**), mimetics of the monomeric unit of the polymers were synthesized and their properties as glycosidase inhibitors were evaluated. The preparation of these derivatives was accomplished through CuAAC reaction of pyrrolidine alkynes **6**, **9**, **12**, **14** and **16** with 3-azidopropyl pivalate [51] in ^{t}BuOH:H_2O using $CuSO_4$ and sodium ascorbate (NaAsc) (Scheme 3).



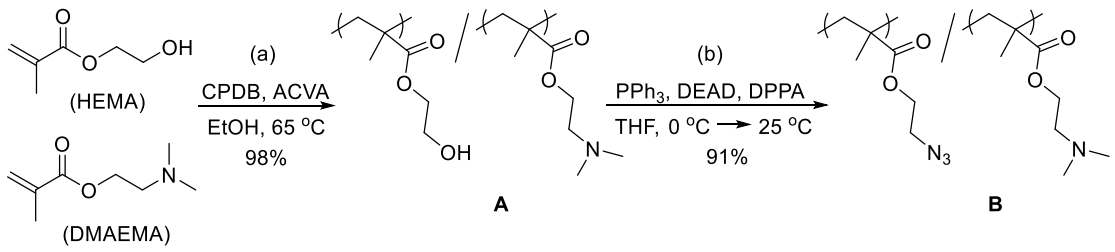
Scheme 3. Preparation of substituted pyrrolidin-triazoles **17-21**

The biological evaluation of compounds **6**, **8** and **9** and **12-21** towards eleven commercial glycosidases (α-fucosidase from bovine kidney, α-galactosidase from coffee beans, β-galactosidases from *A. oryzae* and *E. coli*, α-glucosidases from yeast and rice, β-glucosidase from almonds, β-*N*-acetylglucosaminidase from Jack beans, amyloglucosidase from *A. niger*, α-mannosidase from Jack beans and β-mannosidase from snail) was carried out (Table S1). Compounds **8** and the previously described **13** [38] showed a weak inhibition towards α-mannosidase (65 and 71% at 1 mM concentration of inhibitor, respectively) but their *N*-alkylated analogues **9** and **14** and the corresponding click products **17** and **18** showed no significant inhibition towards any of the enzymes tested. The strong inhibition showed by triazole derivative **15** (97% at 1mM concentration of inhibitor) [38] towards β-glucosidase decreased to 60% in the corresponding *N*-alkylated derivative **16** and was completely abolished in the click compound **19**. Alkynyl pyrrolidine **6** presented a weak-to-moderate inhibition of α-fucosidase (69%) and α-mannosidase (81%), and the corresponding click derivative **20** showed strong inhibition of α-fucosidase (93%). Finally, triazole alkyne **12** and its corresponding click compound **21** showed moderate inhibition of β-glucosidase (83 and 73%, respectively). In view of these results, we decided to choose alkynes **6** and **12** for derivatization of the polymers.

3.3. Synthesis and characterization of the new functionalized polymers

Once the inhibition studies of the prepared iminosugars against selected commercial enzymes were carried out, the preparation of azide-functionalized water-soluble polymeric platforms were conducted next. In the synthesis of the polymeric scaffold for anchoring the iminoglycosides, the reliable and "living" polymerization procedure, Reversible Addition−Fragmentation Chain-Transfer (RAFT) polymerization technique, was chosen [52]. The hydrophilic monomers selected were 2-hydroxyethyl methacrylate (HEMA) and 2-(*N*,*N*-dimethylamino)ethyl methacrylate (DMAEMA) to guarantee the biocompatibility of the resulting copolymer. The non-toxic and biocompatible hydrophilic character of pHEMA is well known what makes it particularly attractive for biomedical engineering applications [53]. PDMAEMA is another biocompatible polymer with pH responsive behavior [54] which has been selected in some formulations for the co-delivery of paclitaxel and DNA [55]. This polymer forms part of *graft*-copolymers or *block*-copolymers with other biocompatible blocks such as polycaprolactone (PCL) or polyethylene glycol (PEG). The pDMAEMA blocks, with tertiary amine groups, is partially protonated at neutral pH [56].

The functionalized polymer with azide groups has been prepared through reactions shown in Scheme 4. A pH-sensitive copolymer with 50% mole composition on each monomer, HEMA and DMAEMA, was aimed.



Scheme 4. General scheme for the synthesis of (**a**) pHEMA-based copolymer by RAFT polymerization (polymer **A**); (**b**) preparation of the functionalized polymer with azide groups (polymer **B**).

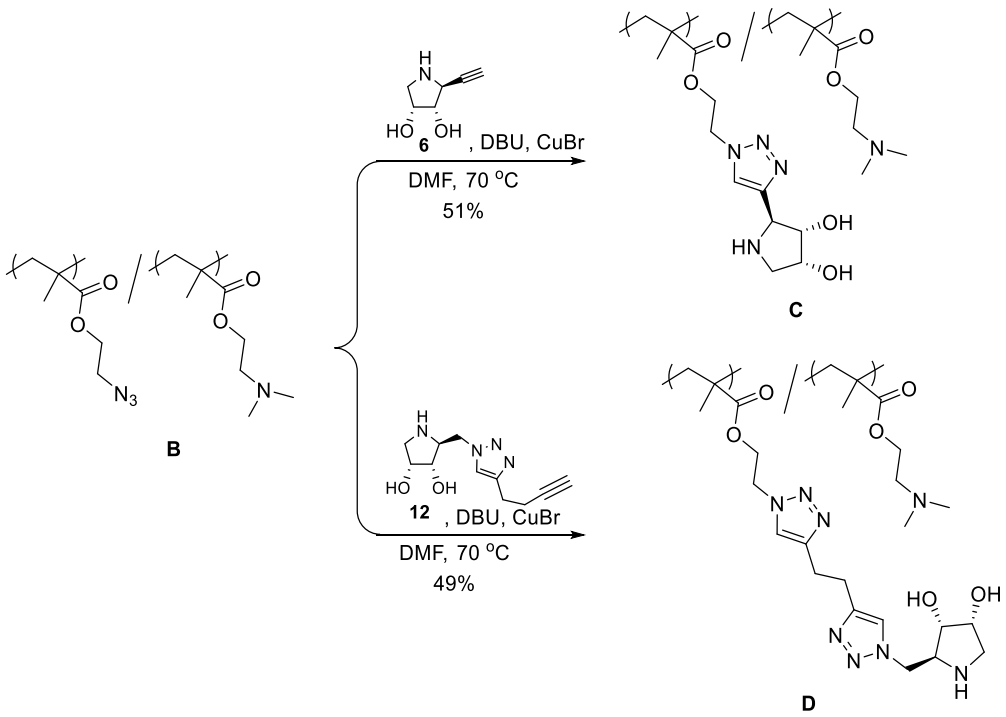
The HEMA-based copolymer (polymer **A**) was obtained by RAFT polymerization in the presence of 2-cyano-2-propyl-4-cyanobenzodithioate (CPCB) as chain transfer agent (CTA) and 4,4'-azobis(4-cyano)pentanoic acid (ACVA) as initiator, following the reported procedure [46] in excellent yield. The values of molecular weights and polydispersity were calculated by gel permeation chromatography (GPC, M_n = 19,800; M_w = 23,800; M_w/M_n = 1.2). The polydispersity or heterogeneity index (M_w/M_n) for this polymer was 1.2, which indicates great homogeneity of

molecular weights. The reliability of the procedure in achieving a controlled copolymer composition was confirmed by ^{1}H NMR. It is remarkable the adjustment in mole compositions between the experimental and the theoretical values (experimental copolymer mole composition: pHEMA$_{49\%}$-*co*-pDMAEMA$_{51\%}$).

The resulting polymer composition did impart not only a hydrophilic character to the material [54,57] but also, and due to the presence of the hydroxyl functional groups (in HEMA moieties), enabled its functionalization by the Mitsunobu reaction towards the azido-derivatived unit 2-azidoethyl methacrylate (AEMA)

The transformation of HEMA units of polymer **A** into 2-azidoethyl methacrylate unit (AEMA) was carried out in the presence of triphenylphosphine, diethyl azodicarboxylate (DEAD) and diphenylphosphorylazide (DPPA) using THF as reaction solvent. GPC and ^{1}H-NMR analyses of the obtained polymer **B** drew the following data: M_n = 19,900, M_w = 30,500, being the polydispersity (M_w/M_n) of the polymer 1.5. ^{1}H NMR experiments confirmed the quantitative conversion of the hydroxyl groups of HEMA units into azido groups leading to a polymer with a final mole composition pAEMA$_{49\%}$-pDMAEMA$_{51\%}$. The peak corresponding to protons "d" from HEMA units (COOCH$_2$CH$_2$) at 3.8 ppm was missing as can be seen in ^{1}H NMR spectrum corresponding to polymer **A** (Supporting Information). Moreover, a broad singlet at 3.48 corresponding to protons "d" from AEMA (COOCH$_2$CH$_2$) was evident in the ^{1}H NMR spectrum of polymer **B** (see Supporting Information). The incorporation of azide groups into the polymer structure was also corroborated in the IR spectrum by the appearance of a band at 2103 cm^{-1} corresponding to the stretching of -N$_3$ group (Figure 3). Likewise, it was observed the disappearance of the band at 3354 cm^{-1} in the IR spectrum of polymer **B** due to the stretching band of O-H bonds in HEMA units.

The selected alkynyl iminocyclitols **6** and **12** were made react with the copolymer pAEMA$_{49}$-pDMAEMA$_{51\%}$ by CuAAC reaction in the presence of CuBr and 1,8-diazabicyclo[5.4.0]undeca-7-ene (DBU) to stabilize Cu(I) in DMF at 70 ^{o}C, furnishing the corresponding copolymers with moderate yields (Scheme 5).



Scheme 5. Preparation of iminoglycopolymers by CuAAC reaction

Both reactions proceeded with complete conversions, a fact confirmed by IR. Thus, in the glycopolymers IR spectra, it is clear the disappearance of the band at 2103 cm^{-1} corresponding to the azido group present in the starting copolymer and used as reaction-limiting reagent (Figure 3). Though the complexity of ^{1}H NMR spectra of polymers **C** and **D**, the disappearance of the singlet at 3.48 ppm from AEMA units as well as the appearance of the peak due to the triazole protons at ca. 8 ppm were evident in both systems.

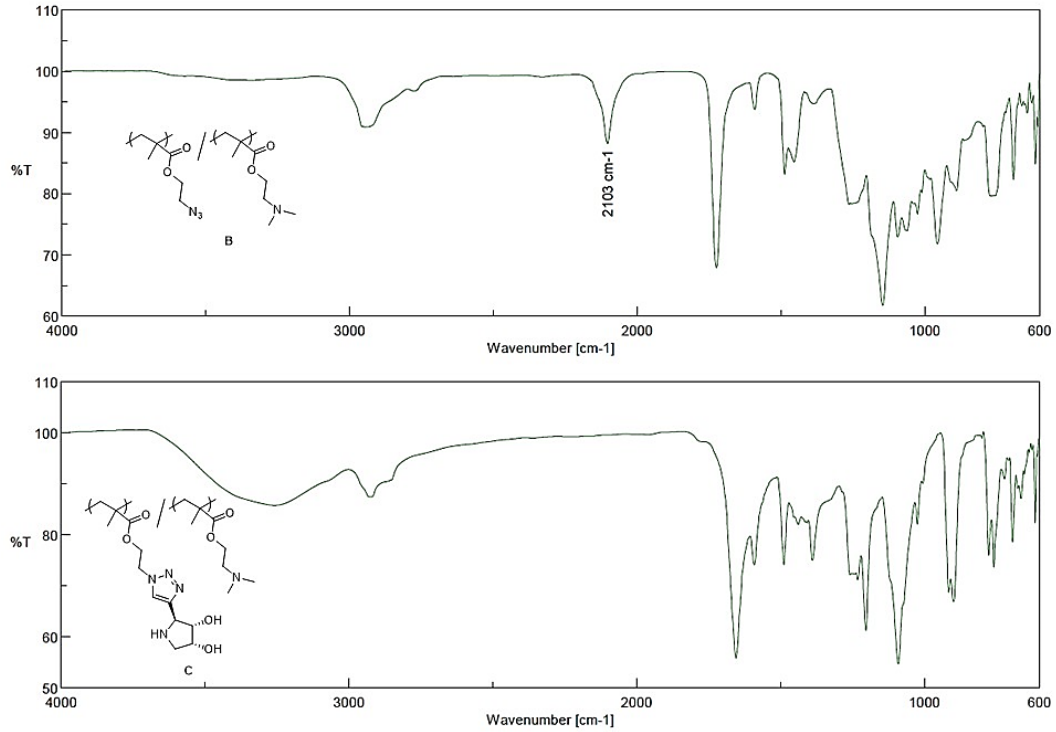


Figure 3. IR spectra of the copolymers **B** (top) and **C** (bottom).

The macromolecules studied in the present work (from Polymer **A** to Polymer **D**), with DMAEMA units, contain tertiary amine groups pendant in the polymer backbone that confer pH-responsive properties to polymeric materials, as demonstrated in previous works [58,59]. To verify if the click reactions altered that feature in the derivatized polymers **C** and **D**, contact angles (CA) were measured for both samples under neutral and acidic conditions. Figure S1 (Supplementary Information) displays a cross-sectional view of a water droplet for each sample before and after the application of phosphoric acid treatment. Treatment with phosphoric acid led to substantial CA decreases (Polymer **C**: from 39.2 º to 22.5 º; Polymer **D**: from 48.4 º to 26.9 º), which confirmed the maintenance of their response capacity when lowering pH.

The thermal properties of the new polymeric materials studied by DSC and TGA are summarized in Table 1.

Table 1: Thermal properties of the synthesized pDMAEMA-based copolymers.

Polymer		DSC[a]	TGA[b]		
		T_g (ºC)	$^{o}T_d$ (ºC)	$^{max}T_d$ (ºC)	ΔW (%)
A	$pHEMA_{49\%}$-$pDMAEMA_{51\%}$	62	251	282/**434**	37/**59**
B	$pAEMA_{49\%}$-$pDMAEMA_{51\%}$	52	239	**278**/403	**42**/39
C	$p(TEMA\text{-}6)_{49\%}$-$pDMAEMA_{51\%}$	-	193	**229**/284/400	**32**/25/12
D	$p(TEMA\text{-}12)_{49\%}$-$pDMAEMA_{51\%}$	-	249	**304**/409	**41**/13

[a]Glass transition temperature (T_g) measured by DSC;
[b]Onset decomposition temperature corresponding to 10% of weight loss ($^{o}T_d$), maximum rate decomposition temperatures; ($^{max}T_d$) and weight loss at the respective decomposition step [ΔW(%)] determined by TGA

Glass transitions (T_g) took place at well-defined temperatures, with values that were highly dependent on the polymer constitution. Thus, the HEMA and AEMA-based copolymers (polymers **A** and **B**) exhibited an amorphous structure with T_g values of 62 °C and 52 °C, respectively. Regarding their thermal stability, the TG profiles (weight loss *vs*. temperature) of **A**, **B**, **C** and **D** are superimposed in Figure 4.

In the TG curves of polymers **A**, **C**, and **D** the presence of residual solvents was found. The TG profiles of HEMA and AEMA-based copolymers (**A** and **B**, respectively) showed two-step degradation processes. For polymer **A**, the second and main step occurred at 434 °C, with an associated weight loss of 59%, whereas polymer **B** experiences the highest weight loss (42%) at 278 ºC. Additionally, the functionalization with the azido moieties of polymer **B** conducted to a reduction in its stability, compared to the starting polymer **A**, leading to an onset decomposition temperature ($^{o}T_d$) of 239 ºC. A reduction in the overall weight loss (up to 700 ºC) under inert atmosphere was found when the incorporation *via* click chemistry of the selected iminosugars (either **6** or **12**) into the polymer backbone were accomplished. Polymer **C** was the glycopolymer with the highest thermal degradability at lower temperatures ($^{o}T_d$ shifted from 239 °C to 193 °C), highlighting the variation in thermo-sensitive properties between the new iminoglycopolymer and its azido-based counterpart.

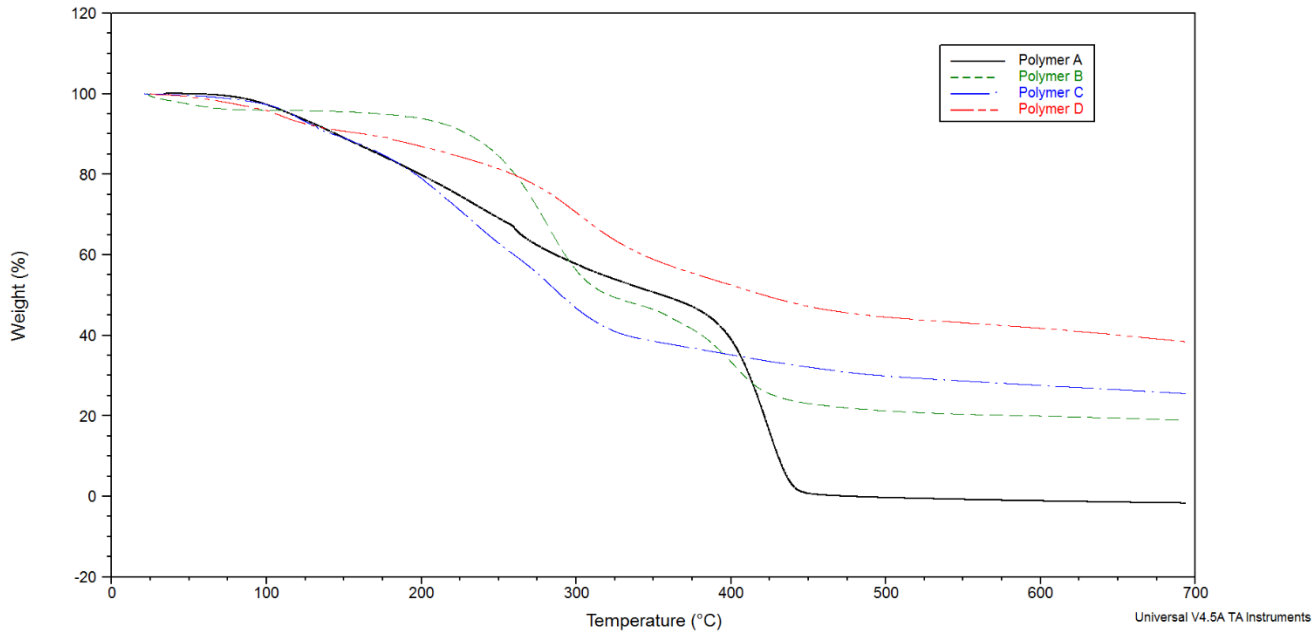


Figure 4. Comparative thermal degradation curves of DMAEMA-based copolymers under inert atmosphere.

Copolymers **C** and **D** [p(TEMA-**6**)$_{49\%}$-pDMAEMA$_{51\%}$ and p(TEMA-**12**)$_{49\%}$-pDMAEMA$_{51\%}$, respectively] were evaluated towards the panel of glycosidases used in the previous section. The prepared imino-glycopolymers have proved to be inactive against the commercial glycosidases tested. However, as the range of enzymes used is limited, the new imino-glycopolymers prepared constitute a new type of copolymers containing *N*-unsubstituted pyrrolidine ring. The applicability of these new materials is currently under study, concerning their activity against other proteins.

4. Conclusions

A robust method has been designed to prepare azide-functionalized biocompatible polymethacrylates, able to anchor selected alkynyl-derivatized iminosugars through CuAAC reaction. The pHEMA-based starting polymer was successfully synthesized through RAFT polymerization with excellent control of the copolymer composition and, subsequently fully functionalized with azide groups *via* one-pot Mitsunobu reaction. Two alkynyl pyrrolidine iminosugars that showed inhibitory activity towards α-fucosidase or β-glucosidase were selected. Several structural requirements concerning the pyrrolidine-alkyne such as the spacer and the position of the linker were considered. The incorporation of biologically-active five-membered

alkynyl iminosugars to the azide-based copolymer was accomplished successfully through CuAAC click reaction leading to the desired water-soluble glycopolymers.

The biological screening towards eleven commercial glycosidases showed that the multimeric presentation of inhitopes in the glycopolymer abolished the activity of the corresponding monovalent compounds.

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Figure Captions

Figure 1. Pyrrolidine triazole hybrid molecules and multivalent assemblies.

Scheme 1. General strategy for the preparation of iminoglycopolymers *via* CuAAC.

Figure 2. Alkyne derivatives for iminosugar anchoring to the polymer.

Scheme 2: Preparation of iminosugars **6**, **9**, **12**, **14** and **16** from D-mannose.

Scheme 3. Preparation of substituted pyrrolidin-triazoles **17-21.**

Scheme 4. General scheme for the synthesis of (**a**) pHEMA-based copolymer by RAFT polymerization (polymer **A**); (**b**) preparation of the functionalized polymer with azide groups (polymer **B**).

Scheme 5. Preparation of iminoglycopolymers by CuAAC reaction.

Figure 3. IR spectra of the copolymers **B** (top) and **C** (bottom).

Figure 4. Comparative thermal degradation curves of DMAEMA-based copolymers under inert atmosphere.

Tables

	Polymer	**DSC[a]**	**TGA[b]**		
		T_g (°C)	**$^{o}T_d$ (°C)**	**$^{max}T_d$ (°C)**	**ΔW (%)**
A	$pHEMA_{49\%}$-$pDMAEMA_{51\%}$	62	251	282/**434**	37/**59**
B	$pAEMA_{49\%}$-$pDMAEMA_{51\%}$	52	239	**278**/403	**42**/39
C	$p(TEMA\text{-}6)_{49\%}$-$pDMAEMA_{51\%}$	-	193	**229**/284/400	**32**/25/12
D	$p(TEMA\text{-}12)_{49\%}$-$pDMAEMA_{51\%}$	-	249	**304**/409	**41**/13

[a]Glass transition temperature (T_g) measured by DSC;
[b]Onset decomposition temperature corresponding to 10% of weight loss ($^{o}T_d$), maximum rate decomposition temperatures; ($^{max}T_d$) and weight loss at the respective decomposition step [ΔW(%)] determined by TGA

Table 1: Thermal properties of the synthesized pDMAEMA-based copolymers.