Versatile Grafting of Polysaccharides in Homogeneous Mild Conditions by Using Atom Transfer Radical Polymerization

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A versatile atom transfer radical polymerization (ATRP) method for polysaccharide grafting in homogeneous mild conditions without using protecting group chemistry is presented. Water/DMF mixtures with different compositions were used as the solvent. The "grafting-from" approach was used in order to prepare suitable pullulan and dextran ATRP macroinitiators with a well controlled degree of functionalization. Methacrylate and acrylamide monomers were grafted obtaining good control over the number, molecular weight and polydispersity of the grafted chains without homopolymer formation and polysaccharide degradation. The versatility of this method allowed us to prepare comblike derivatives with a wide range of properties (amphiphilic, ionic, and thermoresponsive) by simply changing the solvent composition and the catalyst. This could make possible the synthesis of new interesting biomaterials starting from a wide range of polysaccharides.

Introduction

Polysaccharides are biopolymers abundant in nature, usually available at low cost, and intrinsically biodegradable and biocompatible. Applications of polysaccharides range from the biomedical field (drug release systems),[1,2] resorbable materials for surgery and chemotherapy,[3] and tissue engineered devices[4] to the textile and laundry detergent industry[5,6] and to food and cosmetic formulations.[7,8] Chemical modification is often required to modify properties such as solubility, biodegradability, chemical or thermal stability, and mechanical behavior. Grafting of polymers on polysaccharides has been widely used as one of the most convenient ways to combine the advantages of natural and synthetic macromolecules.[9]

Ceric ion initiation, Fenton's reagent, and γ-radiation are among the most widely used methodologies to graft synthetic polymers onto polysaccharides.[1,2,5,7,10−12] Drawbacks of these procedures are the production of unwanted homopolymer together with the graft copolymer, as a consequence of nonspecific initiation and/or chain transfer and the possible degradation of the polysaccharidic backbone with a significant molecular weight decrease. Additionally, these techniques allow poor control over the composition of the graft copolymer (i.e., number, molecular weight, and polydispersity of the grafted chains) and, consequently, on the properties of the final material. In contrast, the recently introduced controlled/"living" radical polymerization techniques (CRP) are known to be able to minimize chain transfer and to control molecular weight and polydispersity.[13−15] In particular, among these methodologies, atom transfer radical polymerization (ATRP)[15] has emerged as a robust synthetic protocol compatible with a wide range of initiators, monomers, and solvents.[15] Nevertheless, only a few papers in the literature reported the use of this technique to modify polysaccharides. For example, Malmström et al. grafted acrylic and methacrylic monomers on insoluble cellulose fibers using surface-initiated ATRP.[16,17] ATRP was also used to polymerize methoxy-capped poly(ethylene glycol methacrylate) in an aqueous suspension of an insoluble chitosan macroinitiator.[18] In another example, *N*-isopropylacrylamide was polymerized by ATRP onto functionalized cross-linked dextran microspheres.[19] All these papers report the functionalization of polysaccharides in heterogeneous conditions because of the poor solubility of most polysaccharides in traditional ATRP solvents. Other authors used ATRP after hydrophobically modifying or protecting carbohydrates and polysaccharides.[20−22] Recently Narain and Armes reported the synthesis by ATRP of novel sugar methacrylate-based polymers without recourse to protecting group chemistry. They ATR polymerized 2-gluconamidoethyl methacrylate (GAMA) and 2-lactobionamidoethyl methacrylate (LAMA) at 20 °C in methanol or water/methanol mixtures.[23−25] However, this method cannot be used for polysaccharides as they are not soluble in alcohols.

N,*N*-Dimethylformamide (DMF) is an interesting solvent as it is able to solubilize many synthetic hydrophilic and hydrophobic polymers as well as a large number of polysaccharides. Unfortunately, DMF has been recognized to be problematic for ATRP because of possible competing complexation of the metal catalyst or ligand exchange.[26−28] We have recently demonstrated that water/DMF mixtures can be used as solvents to carry out ATRP of methacrylates and acrylamides.[29−31] This method allowed us to polymerize monomers with different polarity by adjusting the water:DMF ratio.

In the present work, we report the well controlled, homogeneous grafting of underivatized polysaccharides with vinyl polymers by ATRP in mild conditions. Pullulan, a linear polysaccharide consisting of (1−6)-α-D linked maltotriosyl units, and dextran, an essentially linear polysaccharide with α(1−6) linked D-glucopyranosyl residues, were used as starting materials. We demonstrate that with this method it is possible to prepare comblike derivatives with a wide range of properties

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(amphiphilic, ionic, and thermoresponsive) by simply changing the solvent composition and the catalyst.

Experimental Section

Materials. 2,2′-Bipyridine (bpy) from Aldrich was recrystallized from ethanol to remove impurities. Tris(2-dimethylaminoethyl)amine (Me_6TREN) was prepared as reported by other authors.[32] 2-Hydroxyethyl methacrylate (HEMA, Aldrich) and methyl methacrylate (MMA, Aldrich) were distilled under reduced pressure. *N*-Isopropylacrylamide (NIPAAM, Aldrich) was recrystallized from hexane. 3-Sulfopropyl methacrylate potassium salt (SPMA, Aldrich), ethyl α-bromoisobutyrate (EB*i*B), 2-bromoisobutyryl bromide (BrB*i*B), and all other reagents (from Aldrich) were used as received. Pullulan (PF-20 by Hayascibara CO. LTD, Japan, viscosity average molecular weight $M_v = 1.7 \times 10^5$), dextran (T-70, Amersham-Pharmacia, Uppsala, Sweden, number and weight average molecular weights 61 000 and 106 000 g/mol, respectively), and all other reagents were used as received.

Synthesis of Pullulan-Macroinitiator (Pull-B*i*B$_{DS}$). To obtain a derivative with a degree of substitution (DS, the number of B*i*B groups introduced for 100 glucose units of pullulan) of 5.3%, 2.0 g of pullulan (12 mmol of glucose residues) and 4.4 mmol of 4-(dimethylamino)-pyridine (DMAP) were dissolved in 20 mL of anhydrous DMF at room temperature. A total of 1.2 mmol of α-bromoisobutyryl bromide (B*i*BBr) was added dropwise to the clear ice-cold solution. The mixture was left to warm at room temperature. Aliquots of the reaction mixture were withdrawn at given times and analyzed by FT-IR after isolating the polymer by precipitation in ethanol. The reaction was stopped when the desired DS, as evaluated from the FT-IR spectrum, was obtained. The solution was then diluted with water, filtered, exhaustively dialyzed against distilled water, and freeze-dried. The lyophilized product contained about 12% of water as determined by thermogravimetric analysis. The actual DS was calculated from the ^{1}H NMR spectrum as described in the results and discussion section.

Synthesis of Dextran-Macroinitiators (Dex-2CP$_{DS}$). Reaction conditions were the same described for the synthesis of Pull-B*i*B$_{DS}$, except that 2-chloropropionyl chloride (2CPCl) was used instead of B*i*BBr.

ATRP of HEMA using EB*i*B as the Initiator. Typical polymerization conditions using DMF:H_2O 50:50 (v/v) at 20 °C, [HEMA] = 2 M, [HEMA]:[EB*i*B]:[CuCl]:[$CuCl_2$] = 50:1:1:0.6 and [bpy]/([CuCl] + [$CuCl_2$]) = 2.5 are reported. HEMA (0.781 g, 6.0 mmol), 1.0 mL of water, and 1.5 mL of DMF were introduced in a Schlenk tube and degassed purging with argon. A CuCl−$CuCl_2$−bpy water stock solution was prepared adding 2 mL of degassed water to 47 mg (0.48 mmol) of CuCl, 49 mg (0.29 mmol) of $CuCl_2$, and 300 mg (1.925 mmol) of bpy under argon. After taking an initial sample by syringe to measure the monomer concentration at $t = 0$, degassed EB*i*B (40 mg, 0.12 mmol) and 0.500 mL of the freshly prepared CuCl−$CuCl_2$−bpy stock solution were added to the HEMA solution. The polymerization was sampled at suitable time periods throughout the reaction. Samples for the kinetic study were diluted in water/acetonitrile mixtures and directly injected onto the HPLC columns. GPC analysis was performed by diluting the samples with DMF, filtering the solution through alumina and a 0.45 μm syringe filter, and injecting onto the columns. Number average molecular weights (M_n) were also determined by ^{1}H NMR performing the reaction in fully deuterated solvents and diluting the samples taken at timed intervals in deuterated dimethyl sulfoxide (d_6-DMSO). The number average molecular weight (M_n) was calculated from the ratio of the integral of the pHEMA CH_3 signal to the integral of the initiator CH_3 ethyl signal. When necessary, deconvolution of the peaks was performed.

Grafting of Pullulan with pHEMA (Pull-*g*-pHEMA). In a typical polymerization (DMF:H_2O 50:50 (v/v) at 20 °C, [HEMA] = 1 M, [HEMA]:[Pull-B*i*B$_{5.3}$]:[CuCl]:[$CuCl_2$] = 100:1:1:0.8 and [bpy]/([CuCl] + [$CuCl_2$]) = 2.5), 1.307 g of Pull-B*i*B$_{5.3}$ (0.357 mmol of B*i*B initiating groups) was dissolved with 15.8 mL of water and 17.8 mL of *N*,*N*-dimethylformamide (DMF) in a Schlenk tube and degassed purging with argon. A total of 4.6 g (35.7 mmol) of degassed HEMA was then added. A Cu-bpy stock solution in water was prepared adding 4 mL of degassed water to 35.3 mg (0.357 mmol) of CuCl, 48.7 mg (0.286 mmol) of $CuCl_2$, and 251 mg (1.61 mmol) of bpy under argon. After taking an initial sample by syringe to measure the initial monomer concentration, 2.0 mL of the freshly prepared CuCl−$CuCl_2$−bpy stock solution was added to the HEMA and Pull-B*i*B$_{5.3}$ solution. The polymerization mixture was sampled at suitable time periods throughout the reaction. Kinetic samples were diluted in water/acetonitrile and directly analyzed by HPLC. GPC analysis was performed diluting the samples with DMF, filtering the solutions through alumina and a 0.45 μm syringe filter and injecting onto the GPC columns. The polymerization was stopped by bubbling with air and the product isolated by extensive dialysis against distilled water and lyophilization.

Grafting of Pullulan and Dextran Macroinitiators with Other Monomers. The polymerizations were carried out with similar procedures changing the experimental conditions as follows: (a) $[MMA]_0 = 1.5$ M, $[MMA]_0$:[Pull-B*i*B$_{5.3}$]$_0$:$[CuCl]_0$:$[CuCl_2]_0$:$[bpy]_0$=50:1:1:0.6:4, water:DMF 20:80 (v/v), 50 °C; (b) $[SPMA]_0 = 1.5$ M, $[SPMA]_0$:[Pull-B*i*B$_{5.3}$]$_0$:$[CuCl]_0$:$[CuCl_2]_0$:$[bpy]_0$=50:1:1:1:5, water:DMF 50:50 (v/v), 40 °C. (c) $[NIPAAM]_0 = 2$M, $[NIPAAM]_0$:[Dex-2CP$_{10}$]$_0$:$[CuCl]_0$:$[Me_6TREN]_0$=100:1:1:1, water:DMF 50:50 (v/v), 20 °C.

Isolation of the Grafted Chains by Hydrolysis of the Polysaccharide Backbone. The pullulan backbone of Pull-*g*-pHEMA, Pull-*g*-pMMA, and Pull-*g*-pSPMA derivatives was completely hydrolyzed with 2 M trifluoroacetic acid at 100 °C for 4 h. At the end of the hydrolysis, the mixture was neutralized with NaOH and exhaustively dialyzed (molecular weight cutoff 1000) against distilled water. The polymers were isolated by lyophilization. The hydrolysis of Dex-*g*-pNIPAAM was attempted using the same method but without success. Even using an enzymatic method, the complete hydrolysis of the dextran backbone was not obtained.

Characterization. *NMR Experiments*. Pull-B*i*B (∼3 mg) was solubilized in D_2O (700μL); Pull-*g*-pHEMA (∼3 mg) was also solubilized both in D_2O and in DMSO (700 mL), whereas pHEMA (∼3 mg) was solubilized only in DMSO. ^{1}H and ^{13}C NMR spectra were recorded at 300 K on a Bruker AVANCE AQS600 spectrometer operating at 600.13 and 150.92 MHz, respectively, and equipped with a Bruker *z*-gradient probehead. ^{1}H and ^{13}C assignments were obtained by means of two-dimensional (2D) experiments, namely ^{1}H−^{1}H COSY, ^{1}H−^{1}H TOCSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC experiments.[33] The 2D experiments were carried out using 512 and 1024 data points in the F1 and F2 dimensions, respectively, and a recycle delay of 2 s. The ^{1}H−^{1}H TOCSY experiments were performed with a spin-lock time of 80 ms. The HSQC experiments were performed using a $^1J_{C-H}$ coupling constant of 150 Hz. The HMBC experiments were optimized for a long-range coupling constant of 12 Hz. The number of scans was chosen to achieve a good signal/noise ratio. All 2D experiments were processed with a matrix of 512 × 512 data points. ^{1}H−^{1}H TOCSY and ^{1}H−^{13}C HSQC experiments were processed in the phase-sensitive mode, whereas ^{1}H−^{1}H COSY experiments were processed in the magnitude mode. In the case of Pull-B*i*B solubilized in D_2O, 1D and 2D spectra were recorded using a soft presaturation of the HOD residual signal.[34] To perform a correct integration of minor signals near the HOD residual signal, the ^{1}H spectrum was also performed at 310 K: in this way, it was possible to avoid superimpositions with the HOD signal.

^{1}H and ^{13}C chemical shifts in D_2O were reported in ppm with respect to a trace amount of 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt (DSS) used as an internal standard. ^{1}H and ^{13}C chemical shifts in DMSO-d_6 were obtained referencing to the ^{1}H DMSO residual signal at 2.5 and 40.4 ppm, respectively, from the TMS signal.

Diffusion measurements were performed on dilute solutions (≈1 mg) of PullBr and Pull-p-HEMA in D_2O (700 μL).

Pulsed gradient spin−echo (PGSE) experiments[35] were performed using a pulsed field gradient unit capable of producing a magnetic field

gradient in the *z* direction with a strength of 55.4 G cm^{-1}. The stimulated echo pulse sequence using bipolar gradients with a longitudinal eddy current delay was used.[36] The strength of the sine gradient pulses of 1.4 ms duration was logarithmically incremented in 32 steps, from 2 to 95% of the maximum gradient strength, with a diffusion time of 700 ms and a longitudinal eddy current delay of 25 ms.

After Fourier transformation, phase and baseline correction, the diffusion dimension was processed using the Bruker Xwinnmr software package (version 3.5).

HPLC Experiments. Determination of the monomer conversion was performed using two LabFlow 4000 HPLC pumps (LabService Analytica, Bologna, Italy) equipped with a Knauer K-2501 UV detector (λ = 220 or 230 nm) and a C18 (Phenomenex Luna, 5 μm) column. Different eluents were used for each monomer. The injection volume was 20 μL. DMF was used as internal standard.

Gel Permeation Chromatography (GPC). Molecular weight distributions were obtained using a gel permeation chromatography (GPC) system equipped with a LabFlow 4000 HPLC pump and a Shimadzu RID-10A refractive index detector (Shimadzu, Kyoto, Japan). For aqueous GPC, two or three TSK-GEL GM-PW (30 × 7.5 mm, 17 μm) columns were used. The eluent was 0.2 M NaCl at a flow rate of 0.8 mL/min at room temperature. Near-monodisperse pullulan standards were used for calibration. The concentration of the polymeric solutions was 0.5−1 mg/mL. The injection volume was 20 μL. For the analysis of pHEMA, pMMA, Pull-*g*-pHEMA, and Pull-*g*-pMMA, two or three Polymer Laboratories PLgel Mixed-B (10 μm) columns were used. The eluent was DMF containing 1% triethylamine and 1% glacial acetic acid at a flow rate of 0.8 mL/min. The temperature of the column was 40 °C. Near monodisperse polystyrene (Shodex) and pMMA (Polymer Laboratories) standards were used for the analysis of pHEMA and pMMA, respectively. Pullulan standards (Shodex) were used for the analysis of Pull-*g*-pHEMA and Pull-*g*-pMMA. In some cases, GPC calibration was performed using pHEMA standards prepared by ATRP in our lab, after determining the molecular weight by ^{1}H NMR (see the polymerization of HEMA with ethyl α-bromoisobutyrate section).

Fourier Transform Infrared Spectroscopy (FTIR). IR spectra were obtained with a Shimadzu 8300 FTIR spectrometer (Shimadzu, Tokyo, Japan) equipped with an ATR Golden Gate accessory (Specac Inc., USA).

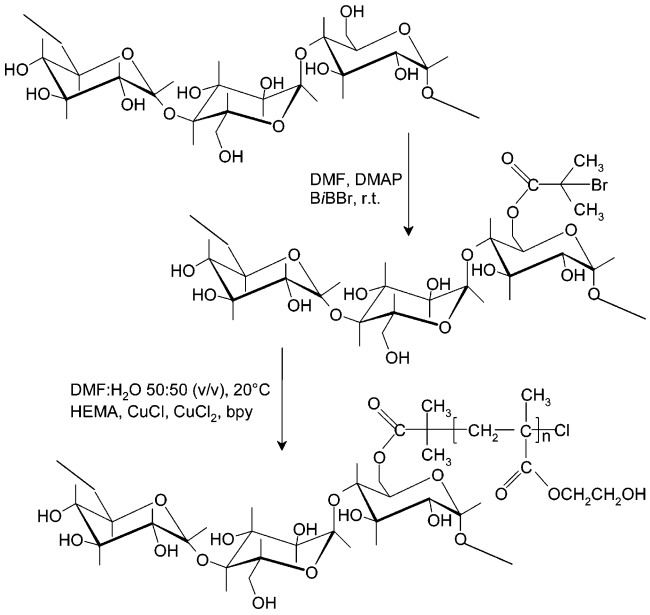
Dynamic Light Scattering (DLS). Data were obtained at 25 °C with a Brookhaven Instruments Corp. BI-200SM goniometer equipped with a BI-9000AT digital correlator using a solid-state laser (125 mW, λ = 532 nm). Measurements of scattered light were normally made at a scattering angle θ of 90°. Some measurements at other angles were made to check the angular dependence of intensity. Experiment duration was in the range of 5−20 min, and each experiment was repeated two or more times.

Results and Discussion

The synthesis of the polysaccharide-graft copolymers was based on the "grafting from" method, which consists of the in situ formation of polymer chains from the substrate, as opposed to the "grafting to" approach, according to which preformed end-functionalized polymers are linked to the substrate. Using the ATRP method, the "grafting from" approach involves two steps: the introduction of ATRP initiating groups on the polysaccharidic backbone (macroinitiator synthesis) and the subsequent graft polymerization. As an example, the procedure used for the synthesis of pullulan derivatives grafted with pHEMA is reported in Scheme 1.

Synthesis of the Macroinitiators. The Pullulan macroinitiator (Pull-B*i*B$_{DS}$) was obtained by partial esterification of the hydroxyl groups of the polysaccharide with α-bromoisobutyryl bromide (B*i*BBr) in the presence of 4-(dimethylamino)pyridine (DMAP). The reaction was performed in DMF at ambient

Scheme 1. Synthesis of Pullulan Grafted with pHEMA by ATRP



temperature. Macroinitiators having different B*i*B degrees of substitution (DS, the number of B*i*B groups introduced for 100 glucose units of pullulan) were prepared by reacting pullulan with different amounts of B*i*BBr. The formation of the ester bond resulted in the appearance of the C═O stretching band (1730 cm^{-1}) in the FTIR spectrum (Figure 1). The intensity of this band increases with the amount of B*i*BBr used in the reaction. The stretching of the polysaccharide hydroxyl groups is also visible in the FTIR spectrum (3300 cm^{-1}) and decreases with the increasing degree of substitution, although not proportionally.

The functionalization of pullulan with the initiator was carefully studied by NMR. The assignment of the ^{1}H and ^{13}C spectra of the Pull-B*i*B derivative with the higher DS was obtained by means of ^{1}H−^{1}H COSY, ^{1}H−^{1}H TOCSY, ^{1}H−^{13}C HMQC, and ^{1}H−^{13}C HMBC experiments performed at 300 K. The HMQC map of a sample of Pull-B*i*B dissolved in D_2O is shown in Figure 2; the corresponding ^{1}H and the ^{13}C NMR spectra are reported as projections in the same figure. Besides the resonances of the unsubstituted pullulan, other resonances are present. An accurate study of the HMQC map and considerations on chemical shift values allowed units substituted in positions 6, 3, and 2 to be assigned (the criterion

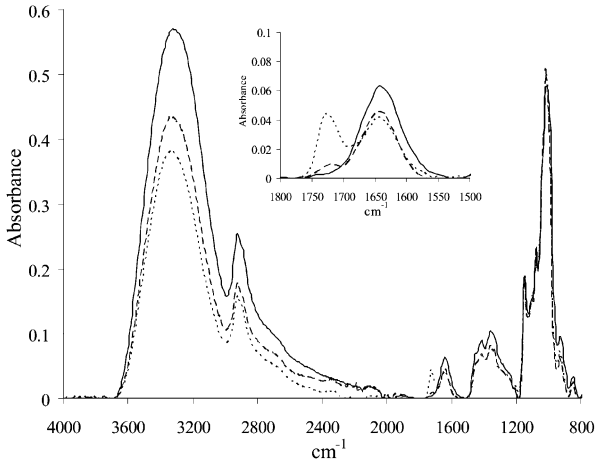


Figure 1. FTIR spectra of pullulan (—), Pull-B*i*B$_{1.0}$ (− − −), and Pull-B*i*B$_{5.3}$ (- - -).

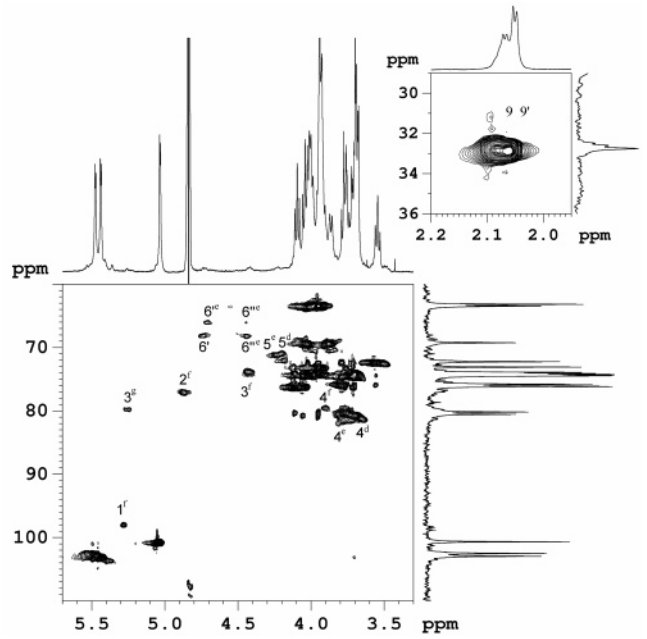


Figure 2. $^{1}H-^{13}C$ HMQC map of a sample of Pull-B*i*B$_{5.3}$ at 300 K. In the inset, the methyl region is reported. ^{1}H and ^{13}C spectra are reported as projections. The assignment of the substituted units is also reported according to the notation reported in Table 1.

used to indicate the different units is explained in the notation of Table 1). The presence in the HMQC map of cross-peaks between ^{1}H resonances at 4.735 and 4.434 ppm and the ^{13}C resonance at 68.11 ppm and between ^{1}H resonances at 4.694, 4.438 ppm and ^{13}C resonance at 65.93 ppm suggest the presence of two CH_2 belonging to units substituted in position 6, reported according to our notation as d and e units. In fact, due to the presence of a carbon atom in a β position, the ^{13}C chemical shifts of C6 in units substituted in positions 6 are downfield shifted with respect to the chemical shifts of C6 of unsubstituted units at about 63 ppm. Therefore, two units substituted in position 6 are identified and, according to our notation, the ^{1}H resonances are reported as H6′^{d}, H6″^{d} and as H6′^{e}, H6″^{e} with the corresponding carbon resonances reported as C6^{d} and C6^{e}, respectively. From this starting point, the $^{1}H-^{1}H$ TOCSY experiment together with the HMQC experiments allowed the resonances H5^{d}, C5^{d} and H4^{d}, C4^{d} as well as the resonance H5^{e}, C5^{e} and H4^{e}, C4^{e} of these units to be assigned, see Table 1.

The presence of an anomeric carbon atom at 98.30 ppm upfield shifted with respect to the anomeric resonances of unsubstituted units suggests the presence of a further carbon atom in the γ position and, therefore, a substitution in position 2, reported as f unit. The HMQC map allowed the corresponding proton to be assigned. The COSY and the HMQC experiments allowed the assignment of H2^{f} and C2^{f}. Again, due to the presence of a carbon atom in the β position, the resonance of C2^{f} is observed at 77.00 ppm, definitely downfield shifted with respect to the C2 resonance of unsubstituted units. Other proton and carbon resonances belonging to the same unit were assigned by $^{1}H-^{1}H$ TOCSY and HMQC (Table 1).

Units substituted in position 3 are easily identified through the characteristic ^{1}H and ^{13}C chemical shits of H3^{g} and C3^{g} observed at 5.230 and 80.00 ppm, respectively; again, due to the presence of a carbon atom in the β position, the resonance of C3^{g} is definitely shifted downfield with respect to the C3 resonance of the unsubstituted one. The assignment of other proton and carbon resonances of these units are reported in Table 1.

Table 1. ^{1}H and ^{13}C Assignments of Pull-B*i*B in D_2O at 300 K[a]

	^{1}H (ppm)	^{13}C (ppm)
1[a]	H1[a] 5.033	C1[a] 100.66
2[a]	H2[a] 3.687	C2[a] 73.92
3[a]	H3[a] 4.089	C3[a] 76.07
4[a]	H4[a] 3.749	C4[a] 80.02
5[a]	H5[a] 3.935	C5[a] 73.09
6[a]	H6[a] 3.935	C6[a] 63.24
1[b]	H1[b] 5.469	C1[b] 103.01
2[b]	H2[b] 3.710	C2[b] 74.24
3[b]	H3[b] 4.033	C3[b] 76.03
4[b]	H4[b] 3.688	C4[b] 80.58
5[b]	H5[b] 3.937	C5[b] 74.03
6[b]	H6[b] 3.985	C6[b] 63.45
1[c]	H1[c] 5.432	C1[c] 102.96
2[c]	H2[c] 3.692	C2[c] 74.24
3[c]	H3[c] 3.744	C31[c] 75.82
4[c]	H4[c] 3.538	C4[c] 72.24
5[c]	H5[c] 4.004	C5[c] 74.21
6′[c]	H6″[c] 3.867	C6[c] 69.22
6″[c]	H6″[c] 4.021	C6[c] 69.22
7		176.11
8		59.42
9, 9′	2.044, 2.062	32.72
4[d]	H4[d] 3.722	C4[d] 81.31
5[d]	H5[d] 4.223	C5[d] 71.09
6′[d]	H6′[d] 4.735	C6[d] 68.11
6″[d]	H6″[d] 4.434	C6[d] 68.11
4[e]	H4[e] 3.748	
5[e]	H5[e] 4.180	C5[e] 71.881
6′[e]	H6′[e] 4.694	C6[e] 65.94
6″[e]	H6″[e] 4.438	C6[e] 65.94
1[f]	H1[f] 5.254	C1[f] 98.30
2[f]	H2[f] 4.853	C2[f] 77.00
3[f]	H3[f] 4.410	C3[f] 73.68
4[f]	H4[f] 3.891	C4[f] 79.47
5[f]	H5[f] 3.985	h[b]
1[g]	H1[g] 5.507	h
2[g]	H2[g] 3.965	h
3[g]	H3[g] 5.237	C3[g] 80
4[g]	H4[g] 3.832	h
5[g]	H5[g] 4.091	h

[a] See Scheme 1. Chemical shifts are reported in ppm with respect a trace of DSS used as internal standard. a, b, c = units of the unsubstituted pullulan; d and e = units of substituted pullulan in positions 6; f = units of substituted pullulan in position 2; g = units substituted in positions 3. [b] h = hidden.

Resonances of the methyls 9 and 9′ of the B*i*B group are observed at 2.054, 2.069 ppm in the ^{1}H dimension and at 32.72 ppm in the ^{13}C dimension (see inset in Figure 2).

The HMBC experiment allowed the quaternary carbon resonances C8 (C−Br) and C7 (C═O) of the B*i*B group to be assigned at 59.42 and 176.11 ppm, respectively; in Figure 3, an expanded region of the HMBC map is shown, showing the long-range coupling among the methyls 9 and 9′ and the quaternary carbon 8 and the carbonyl carbon 7. By integrating the proper proton resonances, it is possible to obtain the degree of substitution in positions 6, 2, and 3 respectively, as well as the total degree of substitution. In fact, the total DS is easily obtainable by the ratio of the integral of the methyls 9 and 9′ divided by 6 and the integral of H1[a] considered as a reference integral multiplied by 3: the total DS is 5.3%. This value can be verified adding the DS of each unit. The DS in position 6 (units d and e) can be determined by measuring the value of the integral of H6′^{d}, H6″^{d}, H6′^{e}, and H6″^{e} with respect to the reference integral: the DS is 2.1%. The DS in position 2 (f

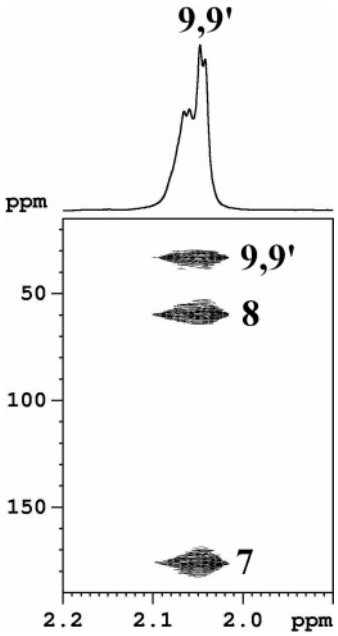


Figure 3. Expansion of the HMBC map of a sample of Pull-B*i*B$_{5.3}$ at 300 K. The long-range correlations among methyls 9 and 9′ and the quaternary carbons 8 and 7 are shown. The assignment is reported according to the notation of Table 1.

unit) can be determined by measuring the integral of H2^{f}; due to the overlapping of this resonance with the residual HOD resonance, the integration was performed on a ^{1}H spectrum at 313 K. The DS is 2.5%. Analogously, the DS in position 3 was determined by measuring the integral of the broad signal at 5.25 ppm due to H^{1f} and H^{3g} and then subtracting the contribution of a proton of the unit substituted in position 2 previously calculated: the DS is 1%.

The same method was used to calculate the DS of the Pull-B*i*B with a lower amount of initiator obtaining DS = 1.0%.

The two macroinitiators were also characterized by GPC (data not shown). The elution profiles of pullulan and Pull-B*i*B macroinitiators were almost identical indicating that no degradation of the polysaccharide takes place as a consequence of the derivatization reaction.

The Pull-B*i*B macroinitiator can be used for the ATRP of methacrylate monomers because the structure of the radical that forms after initiation resembles that of the polymethacrylate propagating chain. For acrylamide monomers, a 2-chloropropionyl (2CP) functionalized macroinitiator was prepared by reacting dextran with 2-chloropropionyl chloride. A dextran macroinitiator with DS = 10% (Dex-2CP$_{10}$) was prepared using the same reaction conditions described for pullulan. GPC experiments demonstrated that no degradation of the polysaccharide occurs during the derivatization.

Model Study of ATRP of HEMA. The first graft polymerization was tried with 2-hydroxyethyl methacrylate (HEMA) because this monomer and its polymer (pHEMA) are soluble in a wide range of water:DMF ratios. The polymerization was preliminarily studied using ethyl α-bromoisobutyrate (EBiB) as the initiator and 2,2′-bipyridine (bpy) as the ligand. In view of the results recently obtained in our laboratory,[29−31] the polymerization was performed in a water:DMF 50:50 (v/v) mixed solvent at room temperature. This solvent was able to dissolve both pullulan and pHEMA. A CuCl/CuCl$_2$/bpy catalyst was used keeping constant (2.5) the ratio of the equivalents of bpy to the total amount of copper species. The mixed halogen initiating system R−Br/CuCl[37−39] and a high amount of Cu(II)[30,31,40,41]

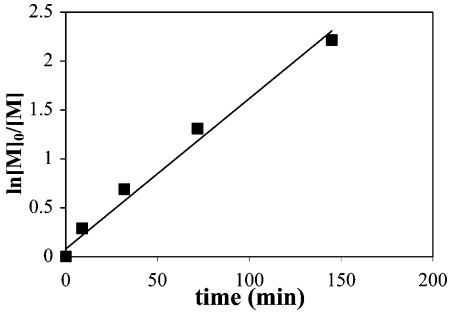


Figure 4. First-order kinetic plot for ATRP of HEMA on Pull-B*i*B$_{5.3}$ macroinitiator in DMF:H$_2$O 50:50 (v/v) at 20 °C. [HEMA] = 1 M. [HEMA]:[Pull-B*i*B$_{5.3}$]:[CuCl]:[CuCl$_2$]:[bpy] = 100:1:1:0.8:4.5.

have been used to improve the control of ATRP. Linear first-order kinetic plots up to very high conversions were obtained for target degrees of polymerization X_n of 50 and 200 (Supporting Information). The molecular weights were in good agreement with the values expected at a given conversion, and the polydispersity index (M_w/M_n) decreased up to 1.17−1.20 (Supporting Information), as expected for a well controlled polymerization.

Grafting of Pullulan with pHEMA. Grafting of pHEMA on Pull-B*i*B$_{5.3}$ was carried out in water:DMF 50:50 (v/v), T = 20 °C, [HEMA]$_0$:[Pull-B*i*B$_{5.3}$]$_0$:[CuCl]$_0$:[CuCl$_2$]$_0$:[bpy]$_0$ = 100:1:1:0.8:4, [HEMA]$_0$ = 1 M. The kinetic plot (Figure 4) showed a reasonable linear dependence of ln([M]$_0$/[M]) with time up to about 90% conversion (145 min), indicating a constant concentration of propagating species and therefore negligible termination. The viscosity of the polymerization mixture increased progressively with the conversion, but no microgels or gel formation were detected. This suggests that termination by radical coupling, which would lead to cross-linking, is absent. Four Pull$_{5.3}$-*g*-pHEMA$_{X_n}$ derivatives with different X_n (25, 49, 75, and 89) were prepared and isolated by stopping the polymerization at different conversions. The ^{1}H NMR spectra in DMSO-d_6 of derivatives having pHEMA grafted chains with X_n = 25 and 89 confirmed the grafting of pHEMA on pullulan (Figure 5). DMSO-d_6 was used as the solvent as it is able to dissolve both pullulan and pHEMA. With respect to the spectrum obtained in D$_2$O, in DMSO-d_6 the signals of the protons of the OH groups of the polysaccharide are present. By keeping constant the intensity of the pullulan signals, the intensity of the peaks ascribed to pHEMA increased with increasing the length of the grafted chains. The HEMA:Glucose molar ratio ($n_{HEMA}/n_{Glucose}$) was obtained from the ratio between the integral I_b of all of the resonances in the 1.35 ÷ 2.2 ppm range due to the CH$_2$(b) of pHEMA (see Figure 5) divided by a factor of 2, and the integral I_{4c} of the resonance at 3.1 ppm due to the proton H4^{c} (see Table 1) of pullulan multiplied by a factor of 3

$$\frac{n_{HEMA}}{n_{Glucose}} = \frac{\frac{I_b}{2}}{3I_{4c}}$$

The values obtained by this method (1.40 and 5.33 for Pull$_{5.3}$-*g*-pHEMA$_{25}$ and Pull$_{5.3}$-*g*-pHEMA$_{89}$, respectively) were in reasonable agreement with the values (1.33 and 4.72) calculated on the basis of the HEMA conversion measured by HPLC.

Note that before integrating the resonance of the H4^{c} a careful baseline correction was required.

Pull$_{5.3}$-*g*-pHEMA$_{25}$ was also analyzed by diffusion ordered NMR spectroscopy (DOSY). Molecular self-diffusion can be

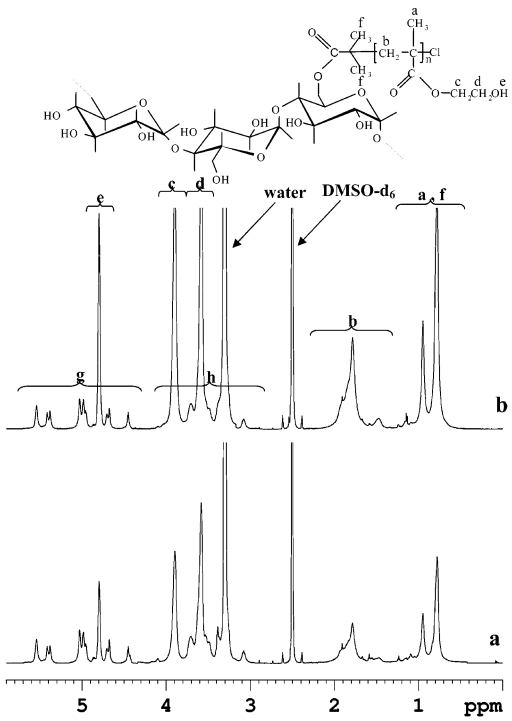


Figure 5. ^{1}H NMR spectra of Pull$_{5.3}$-*g*-pHEMA$_{25}$ (a) and Pull$_{5.3}$-*g*-pHEMA$_{89}$ (b) in d_6-DMSO. The scheme of Pull-*g*-pHEMA is also reported. (g) Anomeric and OH pullulan protons, (h) non anomeric pullulan protons.

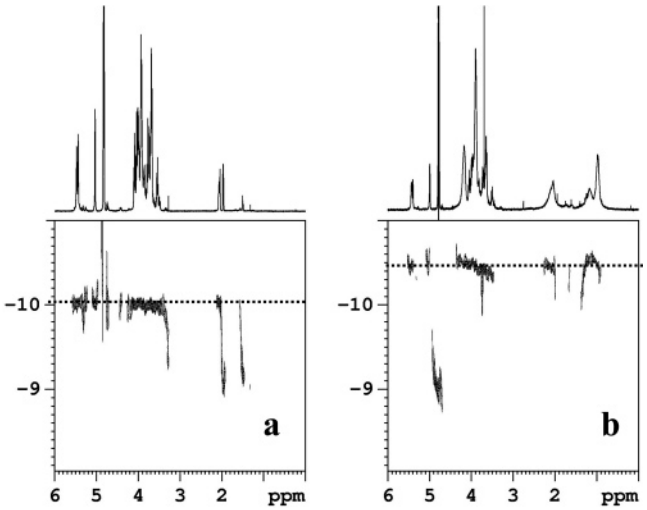


Figure 6. DOSY experiments performed on dilute D_2O solutions at 300 K: Pull-B*i*B$_{5.3}$ (a) and Pull$_{5.3}$-*g*-pHEMA$_{89}$ (b).

encoded into NMR data sets by means of pulsed magnetic field gradients.[35] As first proposed by Morris and Johnson, the diffusion information may be conveniently represented in the form of a two-dimensional map showing the chemical shifts and the diffusion coefficients along the horizontal and vertical axis, respectively, according to DOSY scheme.[36] Basically, DOSY allows compounds to be distinguished according to their diffusion coefficients and therefore to their size; this technique has been used in many applications.[42]

In our case, DOSY experiments were performed on dilute D_2O solutions of Pull-B*i*B$_{5.3}$ and of Pull$_{5.3}$-*g*-pHEMA$_{25}$; the corresponding maps are reported in Figure 6, panels a and b. It is worth mentioning that resonances of methyl 9 and 9′ from the initiator and resonances of pullulan itself show the same diffusion coefficient (Figure 6a). Furthermore, low molecular weight compounds are easily recognized because of their faster diffusion coefficient.

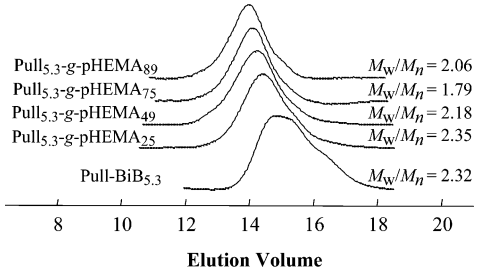


Figure 7. GPC chromatograms of Pull-B*i*B$_{5.3}$ and Pull$_{5.3}$-*g*-pHEMAs.

The DOSY map of Pull$_{5.3}$-*g*-pHEMA$_{25}$ reported in Figure 6b indicates that all the resonances of the pullulan backbone as well as the resonances of the grafted pHEMA, show the same diffusion coefficient. This observation points out that the pHEMA chains are actually grown on pullulan.

The diffusion coefficient of the Pull-B*i*B and Pull$_{5.3}$-*g*-pHEMA$_{25}$ are 9.84×10^{-11} and 3.51×10^{-11} m^2/s, respectively. The lower value for Pull$_{5.3}$-*g*-pHEMA$_{25}$ indicates slower diffusion and therefore, as expected, a higher molecular weight than Pull-B*i*B.

GPC analysis of Pull$_{5.3}$-*g*-pHEMA confirmed that grafting was successful (Figure 7). The GPC chromatograms were obtained in DMF with 1% triethylamine and 1% glacial acetic acid because of the very limited solubility of pHEMA in water. Analysis of pullulan polymer standards under the same conditions demonstrated that this solvent system is suitable to the qualitative analysis of pullulan-based materials. The chromatograms of Pull$_{5.3}$-*g*-pHEMA showed a small but progressive shift of the peak of the derivatives toward higher molecular weights. The small shift observed despite the significant increase in molecular weight can probably be ascribed to the relatively compact structure maintained by the Pull$_{5.3}$-*g*-pHEMA copolymers as a consequence of their branched structure. For the same reason, no attempt to calculate the molecular weights of the grafted derivatives was done. The absence of shoulders or peaks at very high molecular weights suggests that termination reactions by radical coupling are negligible. Additionally, the absence of low molecular weight peaks seems to exclude the formation of HEMA homopolymers.

Hydrolysis of Pull$_{5.3}$-*g*-pHEMA: Characterization of the Grafted pHEMA. Linear evolution of molecular weight with conversion and low polydispersity of the grafted chains are important parameters to demonstrate the controlled character of polymerizations. Polysaccharide chains can be degraded by acid hydrolysis. In the case of pullulan-*g*-pHEMA, backbone hydrolysis leads to the release of the pHEMA grafted chains that can then be isolated and characterized. The four Pull$_{5.3}$-*g*-pHEMA derivatives with different X_n of pHEMA (25, 50, 75, and 89) were hydrolyzed with 2 M trifluoroacetic acid at 100 °C. pHEMAs were isolated by dialysis and freeze-drying and analyzed by GPC (Figure 8). Well-defined monomodal peaks were obtained. The M_n of the purified pHEMA samples were in reasonable agreement with the theoretical values and increased linearly with conversion. Polydispersities decreased from 1.24 to 1.18 with increasing X_n, indicating good control on the polymerization. Analysis of a commercial pHEMA sample after treatment under the same hydrolysis conditions showed no modification of the molecular weight, therefore validating the experimental procedure.

The pHEMA polymers obtained by hydrolysis of Pull$_{5.3}$-*g*-pHEMA were also characterized by ^{1}H and ^{13}C NMR. As an

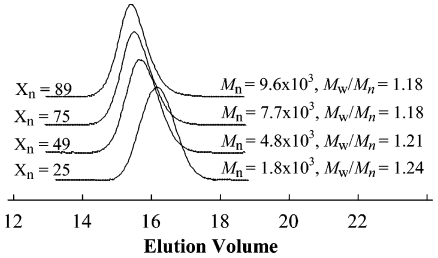


Figure 8. GPC chromatograms of pHEMA chains obtained after the hydrolysis of the Pull$_{5.3}$-*g*-pHEMA copolymers.

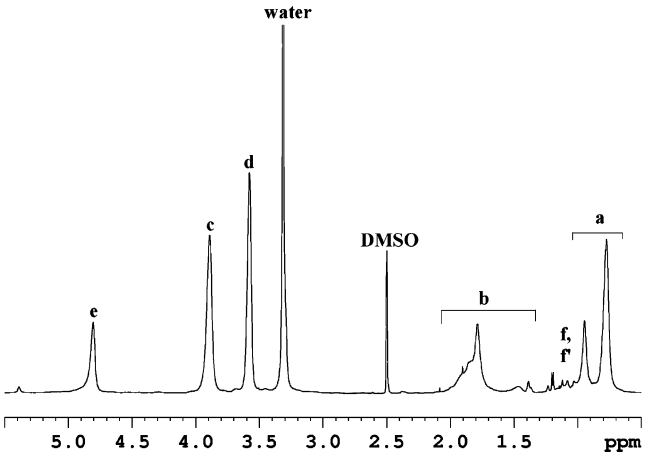


Figure 9. ^{1}H NMR spectrum of pHEMA as obtained by hydrolysis of Pull$_{5.3}$-*g*-pHEMA$_{25}$. See Figure 5 for assignments.

example, the ^{1}H NMR spectrum of pHEMA obtained after hydrolysis of Pull$_{5.3}$-*g*-pHEMA$_{25}$ is shown in Figure 9. HMQC and HMBC experiments allowed us to assign the resonances (data not reported). The multiplicity of resonances a and b is due to tacticity. However, a detailed assignment of diads, triads and so on is beyond the aim of the present work. The spectra confirmed that pHEMA is not altered by the hydrolysis treatment.

Grafting of Other Vinyl Monomers. We recently demonstrated that ATRP in water:DMF mixed solvents is a powerful tool for the direct synthesis of amphiphilic block copolymers containing segments with very different solubility.[29−31] Therefore, we also tried to graft the Pull-B*i*B$_{5.3}$ macroinitiator with methyl methacrylate (MMA) and 3-sulfopropyl methacrylate (SPMA). Grafting with pMMA was carried out in water:DMF 20:80 (v/v). Due to the insolubility of MMA in water, a higher content of DMF was necessary to keep the grafted derivative in solution up to high MMA conversions. The polymerization was carried out using [MMA]$_0$:[Pull-B*i*B$_{5.3}$]$_0$:[CuCl]$_0$:[CuCl$_2$]$_0$:[bpy]$_0$ = 50:1:1:0.6:4. A lower amount of Cu(II) was used because MMA polymerization is slower than that of HEMA. For the same reason, the temperature was 50 °C instead of 20 °C. This temperature was also useful to maintain lower viscosity of the polymerization mixture, especially at high conversions. The first order kinetic plot was linear up to 71% conversion (Figure 10). The solution became very viscous but neither precipitation nor gel formation was observed confirming that the grafted derivative was kept in solution by the higher amount of DMF used in the solvent. Despite the higher temperature and the lower amount of Cu(II), the polymerization was slower than that of HEMA. This is partly due to the higher reactivity of HEMA with respect to MMA. The most significant contribution should be due to the lower percentage of water, which is known to accelerate ATRP due to the hydrolysis of the Cu(II) complex.[40,41,43] The molecular weight ($M_n = 5.0 \times 10^3$) obtained

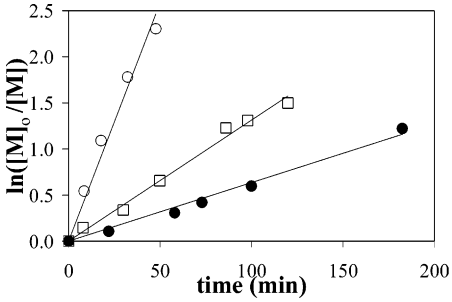


Figure 10. First-order kinetic plot for ATRP grafting using Pull-B*i*B$_{5.3}$ and Dex-2CP$_{10}$ macroinitiators: ●, Pull$_{5.3}$-*g*-pMMA; ○, Pull$_{5.3}$-*g*-pSPMA; □, Dex$_{10}$-*g*-pNIPAAM. Polymerization conditions are described in the text.

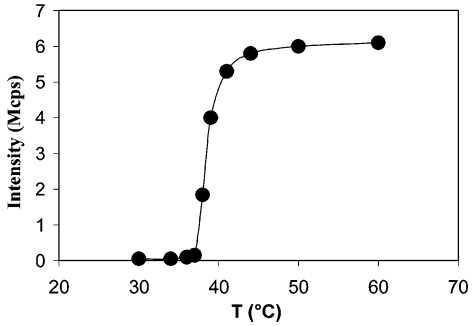


Figure 11. Temperature dependence of the scattered intensity for an aqueous solution (1 mg/mL) of Dex$_{10}$-*g*-pNIPAAM$_{70}$.

by GPC analysis of the pMMA chains obtained by hydrolysis of Pull$_{5.3}$-*g*-pMMA$_{35}$ was in reasonable agreement with the theoretical value. The low value of the polydispersity (1.19) confirmed the control of the polymerization. Preliminary experiments (data not reported) showed that the amphiphilic Pull$_{5.3}$-*g*-pMMA derivatives are able to form well-defined nanoparticles in water.

The grafting of Pull-B*i*B$_{5.3}$ with SPMA was easily obtained using polymerization conditions similar to those used for HEMA (water:DMF 50:50 (v/v), T = 40 °C, [SPMA] = 1.6 M, [SPMA]$_0$:[Pull-B*i*B$_{5.3}$]$_0$:[CuCl]$_0$:[CuCl$_2$]$_0$:[bpy]$_0$ = 50:1:1:1:5). The polymerization was faster than that of MMA and reached 90% conversion in only 48 min. The first-order kinetic plot was linear confirming that termination is negligible and that the polymerization is controlled (Figure 10). Molecular weights and polydispersities of pSPMA obtained by hydrolysis of Pull$_{5.3}$-*g*-pSPMA$_{45}$ determined by GPC confirmed the control of the polymerization. This result demonstrates that by adjusting the solvent composition this method allows to prepare grafted derivatives with an extremely wide range of properties.

Finally, to extend the method to acrylamide monomers, thermosensitive derivatives were prepared using the Dex-2CP$_{10}$ macroinitiator to start *N*-isopropylacrylamide (NIPAAM) polymerization. PolyNIPAAM is a thermoresponsive polymer which is water soluble at room temperature and gives a coil-to-globule transition above 32 °C (the LCST, lower critical solution temperature).[44] We have demonstrated that controlled ATRP of NIPAAM can be obtained using a Me$_6$TREN based catalyst instead of bpy.[29] The grafting was successfully carried out in DMF:water 50:50 (v/v) at 20 °C ([NIPAAM]$_0$ = 2 M, [NIPAAM]$_0$:[Dex-2CP$_{10}$]$_0$:[CuCl]$_0$:[Me$_6$TREN]$_0$ = 100:1:1:1). The first-order kinetic plot was linear up to 78% conversion (120 min) (Figure 10). In this case, we have not been able to isolate pNIPAAM chains by hydrolysis. The thermoinduced association of a grafted derivative with pNIPAAM chains with X_n = 70 (Dex$_{10}$-*g*-pNIPAAM$_{70}$) was preliminary studied

observing the temperature dependence of the intensity of the scattered light measured by dynamic light scattering (DLS) in aqueous solutions. A well-defined transition with a LCST of about 37 °C was observed. This value is higher than the LCST of Dex-*g*-pNIPAAM samples (32−33 °C) obtained by conventional free radical polymerization[45] and is very close to body temperature. The LCST value obtained in our work is in good agreement with the recently reported LCST of low polydispersity pNIPAAM samples of similar molecular weight prepared by ATRP.[46,47] In fact, Xia et al. demonstrated that LCST is very sensitive to the variation of X_n of pNIPAAM samples with low polydispersity, varying from 33 to 43 °C when M_n goes from 3.3 to 33 kDa.[46]

This demonstrates that the synthesis of pNIPAAM grafted copolymers by ATRP allows the LCST to be easily controled only by adjusting the molecular weight of the grafted chains without preparing pNIPAAM copolymers. These results can be very interesting for biomedical applications. Preliminary DLS results showed that the average hydrodynamic diameter of the aggregates is about 92 nm.

In conclusion, we have demonstrated that vinyl polymers can be successfully grafted on polysaccharides in homogeneous mild conditions without using protecting group chemistry obtaining good control on the number, length, and polydispersity of the grafted chains without homopolymerization and polysaccharide degradation. The versatility of this method was demonstrated by preparing ionic, amphiphilic, and thermoresponsive derivatives. In the case of the thermoresponsive copolymers, the control on molecular weight and polydispersity allowed us to introduce an important additional control over the LCST. The same procedure could be used for a wide range of underivatized polysaccharides to prepare new interesting biomaterials.

Acknowledgment. We thank the University "La Sapienza" (Ateneo Funds) for financial support.

Supporting Information Available. First order kinetic plot (full symbols) and conversion (open symbols) for ATRP of HEMA in DMF:H2O 50:50 (v/v) at 20 °C (Figure 1a). Dependence of molecular weights (full symbols) and polydispersities (open symbols) on conversion (Figure 1b). This material is available free of charge via the Internet at http://pubs.acs.org.

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