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Design and Synthesis of *N*-Maleimido-Functionalized Hydrophilic Polymers via Copper-Mediated Living Radical Polymerization: A Suitable Alternative to PEGylation Chemistry

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Abstract: A series of α-functional maleimide polymethacrylates (M_n = 4.1−35.4 kDa, PDi = 1.06−1.27) have been prepared via copper-catalyzed living radical polymerization (LRP). Two independent synthetic protocols have been successfully developed and the polymers obtained in multigram scale, with an 80−100% content of maleimide reactive chain ends, depending on the method employed. A method for the synthesis of amino-terminated polymers, starting from Boc-protected amino initiators, has also been developed, as these derivatives are key intermediates in one of the two processes studied in the present work. The alternative synthetic pathway involves an initiator containing a maleimide unit "protected" as a Diels−Alder adduct. After the polymerization step, the maleimide functionality has been reintroduced by retro-Diels−Alder reaction, by simply refluxing those polymers in toluene for 7 h. These maleimido-terminated materials, poly(methoxyPEG$_{(475)}$) methacrylates and poly(glycerol) methacrylates, differ for both the nature and size of the polymer side branches and showed an excellent solubility in water, a property that made them an ideal candidate for the synthesis of new polymer−(poly)peptide biomaterials. These functional polymers have been successfully employed in conjugation reactions in the presence of thiol-containing model substrates, namely, reduced glutathione (γ-Glu-Cys-Gly) and the carrier protein, bovine serum albumin (BSA), in 100 mM phosphate buffer (pH 6.8−7.4) and ambient temperature.

Introduction

The use of proteins and peptides as human therapeutics is a rapidly expanding area of research. Many factors are responsible for this interest, including the discovery of new peptides and proteins and a better understanding of the mechanism of their action in vivo. These numbers are set to rise as drug discovery molecules emerge from -omics programs. Unfortunately, most peptide-based molecules have properties that are not conducive to oral delivery and, therefore, must be injected at great cost and inconvenience to patients. Despite the progress in this field, the delivery of (poly)peptide-based drugs and other biologically active proteins to their desired targets still remains a major limitation of these new therapeutics. Parenterally administered proteins are rapidly either cleared from circulation by the reticuloendothelial system (kidney, spleen, liver) or metabolized by peptidases and, thus, can rapidly lose their biological activity. Oral delivery is even more problematic, as protein-based therapeutics can be rapidly destroyed by the digestive system.

Conjugation of proteins to poly(ethylene glycol) (PEG), PEGylation, is an increasingly common approach for the chemical modification of a protein, giving rise to several potential beneficial effects.[1−4] A main function of the conjugating polymer is to enlarge the protein size, reducing the clearance of the drug from the body due to renal excretion. Other advantages related to the use of protein−polymer bioconjugates include increased bioavailability and plasma half-lives, decreased immunogenicity, reduced proteolysis, and enhanced solubility and stability.[5] The first examples of PEG−protein drugs were commercialized in the early 1990s,[6] and since then, a number of clinical trials involving polymer−drug conjugates have shown very promising results. As a consequence, a variety of PEG−protein-based drugs are nowadays being introduced into the market, and the field of polymer therapeutics is now rapidly growing.[7] Recent reports indicated that a number of PEG−insulin derivatives have already shown very promising results in clinical trials as pulmonary-delivered peptide therapeutics, and some of them may soon be introduced into the market with

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an estimated business worldwide of $4.6 billion, opening the way for other inhaled protein therapeutics.[8]

Early work in PEGylation concentrated on lysine-specific conjugation, usually leading to statistical multisite attachment. However, the use of a single site attachment leading to one polymer conjugate per protein has obvious advantages. This can be achieved via the reaction of free cysteine residues in proteins, which is an excellent approach for site-specific modification. Where necessary, free cysteine units can be introduced to appropriate positions of polypeptide surfaces either by reduction of disulfide bridges or by genetic engineering modification.[5] In this latter case, PEGylation of the cysteine muteins can afford homogeneously modified mono-PEGylated species that can be readily purified and retain high biological activity. The terminal reactive chain-ends used for these purposes include maleimide, vinyl sulfone, iodoacetamide, and orthopyridyl disulfide units.[3]

The macromolecular structure of the polymer conjugate has also proven to be crucial for the properties of the corresponding bioconjugates. Previous reports indicate, for example, that the use of branched PEG in place of a linear chain can, in some cases, improve a number of properties, such as resistance to proteolysis, to the action of antibodies and resulted in a lower immunogenicity, due to the so-called "umbrella-like" shape of these polymers.[9,10] The molecular weight and molecular weight distribution (MWD) of the polymer conjugates are also important parameters as the MWD of the polymers is reflected in the polydispersity of the peptide−polymer conjugate.[5] We envisaged that the concept of PEGylation could be extended to many functionalized hydrophilic water-soluble polymers obtainable by controlled radical polymerization, featuring a narrow molecular weight distribution and containing appropriate α-terminal reactivity.[11−13] Transition-metal-mediated living radical polymerization (TMM-LRP), often called atom transfer radical polymerization (ATRP), is a technique that allows a great control over the molecular weight distribution and the architecture of polymers. Moreover, it is very tolerant of a variety of functional groups and protic solvents, including water.[14,15] The extreme versatility of this living polymerization process allows tailoring of both the molecular weight and the macromolecular structure, the latter, a factor that can also influence the selectivity of the conjugation reaction. In the present work, we focused our attention toward poly(methacrylates) containing a maleimide terminus. The choice of this reactive chain-end is due to the high reactivity and selectivity of the maleimide toward the cysteine residues present at the protein surface. Indeed, at neutral pH and below, this functionality is reported to react approximately 1000 times faster with thiols than with amines. There has been a preliminary report on the use of a modified pyridyl sulfide with 2-hydroxy ethyl methacrylate which allowed direct conjugation of the polymer to cysteine residues of bovine serum albumin.[16]

In the present work, we report the synthesis and characterization of new α-maleimide-functional polymers and their conjugation with a model peptide and the carrier protein BSA.

Experimental Section

All reactions were carried out using standard Schlenk techniques under an inert atmosphere of oxygen-free nitrogen, unless otherwise stated. Copper(I) bromide (Aldrich, 98%) was purified according to the method of Keller and Wycoff.[17] *N*-(Ethyl)-2-pyridylmethanimine, *N*-(*n*-propyl)-2-pyridylmethanimine,[18] and (2,2-dimethyl-1,3-dioxolan-4-yl)methyl methacrylate[19] were prepared as described earlier and stored at 0 °C under a dinitrogen atmosphere. All other reagents and solvents were obtained at the highest purity available from Aldrich Chemical Co. and used without further purification unless stated.

4,10-Dioxatricyclo[5.2.1.02,6]dec-8-ene-3,5-dione (1): Maleic anhydride (30.0 g, 306 mmol) was suspended in 150 mL of toluene and the mixture warmed to 80 °C. Furan (33.4 mL, 459 mmol) was added via syringe and the turbid solution stirred for 6 h. The mixture was then cooled to ambient temperature and the stirring stopped. After 1 h, the resulting white crystals were collected by filtration and washed with 2 × 30 mL of petroleum ether. Obtained was 44.4 g (267 mmol, 87% yield) of **1** as small white needless. Mp 124−127 °C (dec). IR (neat): $\tilde{\nu}$ = 1857, 1780, 1309, 1282, 1211, 1145, 1083, 1019, 947, 920, 902, 877, 847, 800, 732, 690, 674, 633, 575 cm^{-1}. ^1H NMR (400.03 MHz, $CDCl_3$, 298 K): δ = 3.17 (s, 2H, CH), 5.45 (t, J = 1.0 Hz, 2H, CHO), 6.57 (t, J = 1.0 Hz, 2H, CH_{vinyl}). $^{13}C\{^1H\}$ NMR (100.59 MHz, $CDCl_3$, 298 K): δ = 48.85 (2C, CH), 82.35 (2H, CHO), 137.12 (2C, CH_{vinyl}), 170.04 (2C, CO). Anal. Calcd for $C_8H_6O_4$: C, 57.84; H, 3.64. Found: C, 57.74; H, 3.68. Mass spectrometry (+EI) *m*/*z* (%): 167 [MH^+] (<1), 121 (7), 98 (22), 94 (13), 68 (100).

4-(2-Hydroxyethyl)-10-oxa-4-azatricyclo[5.2.1.02,6]dec-8-ene-3,5-dione (2): The anhydride **1** (2.00 g, 12.0 mmol) was suspended in MeOH (50 mL) and the mixture cooled to 0 °C. A solution of ethanolamine (0.72 mL, 12.0 mmol) in 20 mL of MeOH was added dropwise (10 min), and the resulting solution was stirred for 5 min at 0 °C, then 30 min at ambient temperature, and finally refluxed for 4 h. After cooling the mixture to ambient temperature, the solvent was removed under reduced pressure, and the white residue was dissolved in 150 mL of CH_2Cl_2 and washed with 3 × 100 mL of water. The organic layer was dried over $MgSO_4$ and filtered. Removal of the solvent under reduced pressure furnished an off-white residue that was purified by flash chromatography (CC, SiO_2, 100% ethyl acetate, R_f (**2**) = 0.26) to give **2** (1.04 g, 5.00 mmol, 42% yield) as a white solid. Mp 139−141 °C (dec). IR (neat): $\tilde{\nu}$ = 3472, 1681, 1435, 1405, 1335, 1269, 1168, 1100, 1053, 1013, 959, 916, 875, 850, 807, 722, 705, 654 cm^{-1}. ^1H NMR (400.03 MHz, $CDCl_3$, 298 K): δ = 1.90 (bs, 1H, OH), 2.90 (s, 2H, CH), 3.69−3.72 (m, 2H, NCH_2), 3.76−3.78 (m, 2H, OCH_2), 5.28 (t, J = 0.9 Hz, 2H, CH), 6.52 (t, J = 0.9 Hz, 2H, CH_{vinyl}). $^{13}C\{^1H\}$ NMR (100.59 MHz, $CDCl_3$, 298 K): δ = 41.77 (1C, NCH_2), 47.50 (2C, CH), 60.18 (1C, OCH_2), 81.04 (2C, CHO), 136.60 (2C, CH_{vinyl}), 176.97 (2C, CO). Anal. Calcd for $C_{10}H_{11}NO_4$: C, 57.41; H, 5.30; N, 6.70. Found: C, 57.16; H, 5.37; N, 6.62. Mass spectrometry

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(+EI) *m*/*z* (%): 210 [MH^+] (16), 142 (38), 111 (43), 110 (41), 98 (29), 82 (42), 68 (100).

2-Bromo-2-methyl Propionic Acid 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-8-en-4-yl) Ethyl Ester (3): A solution of the alcohol **2** (2.22 g, 10.6 mmol) and Et_3N (1.60 mL, 11.7 mmol) in 120 mL of THF (the solution remained slightly turbid) was cooled to 0 °C, and a solution of 2-bromo isobutyryl bromide (1.40 mL, 11.1 mmol) in 40 mL of THF was added dropwise (30 min). The white suspension was stirred for 3 h at 0 °C and subsequently at ambient temperature overnight. TLC (SiO_2, 100% ethyl acetate, R_f (**2**) = 0.18, R_f (**3**) = 0.48) revealed the complete disappearance of the starting material (**2**). The ammonium salt was filtered off and the solvent removed under reduced pressure to give a pale-yellow residue that was purified by flash chromatography (CC, SiO_2, petroleum ether/ethyl acetate 1:1, R_f (**3**) = 0.23). Obtained was 3.54 g (9.88 mmol, 93% yield) of **3** as a white solid. Mp 83−85 °C. IR (neat): $\tilde{\nu}$=1733, 1695, 1419, 1395, 1336, 1278, 1157, 1106, 1015, 874, 852, 824, 724, 706, 654, 603 cm^{-1}. ^1H NMR (400.03 MHz, $CDCl_3$, 298 K): δ = 1.86 (s, 6H, CH_3), 2.84 (s, 2H, CH), 3.78 (t, J = 5.3 Hz, 2H, NCH_2), 4.30 (t, J = 5.3 Hz, 2H, OCH_2), 5.23 (t, J = 1.0 Hz, 2H, CHO), 6.49 (t, J = 1.0 Hz, 2H, CH_{vinyl}). $^{13}C\{^1H\}$ NMR (100.59 MHz, $CDCl_3$, 298 K): δ = 30.65 (2C, CH_3), 37.65 (1C, NCH_2), 47.56 (2C, CH), 55.80 (1C, $C(CH_3)_2Br$), 62.26 (1C, OCH_2), 80.91 (2C, CHO), 136.62 (2C, CH_{vinyl}), 171.46 (1C, CO_{ester}), 175.95 (2C, CO_{imide}). Anal. Calcd for $C_{14}H_{16}NO_5$: C, 46.95; H, 4.50; N, 3.91; Br, 22.31. Found: C, 46.88; H, 4.55; N, 3.79; Br, 22.22. Mass spectrometry (+EI) *m*/*z* (%): 360 [MH^+] (5), 358 [MH^+] (5), 292 (13), 290 (13), 151 (6), 149 (6), 210 (13), 191 (28), 124 (67), 123 (57), 110 (41), 69 (65), 68 (100).

2-Bromo-2-methyl Propionic Acid 2-(3,5-Dioxo-10-oxa-4-azatricyclo[5.2.1.02,6]dec-8-en-4-yl) Ethyl Ester (4): Product **3** (0.120 g, 0.335 mmol) was suspended in toluene (5 mL), and the mixture was heated to reflux. The reaction was monitored by TLC (SiO_2, 100% Et_2O, R_f (**3**) = 0.23, R_f (**4**) = 0.46). After 6 h, the solvent was removed under reduced pressure to give **4** (0.095 g, 0.928 mmol, 98% yield) as an off-white solid. Mp 65−66 °C. IR (neat): $\tilde{\nu}$ = 3099, 2975, 1698, 1442, 1404, 1404, 1387, 1368, 1329, 1269, 1152, 1103, 1041, 984, 925, 834, 761, 693, 645 cm^{-1}. ^1H NMR (400.03 MHz, $CDCl_3$, 298 K): δ = 1.88 (s, 6H, CH_3), 3.84 (t, J = 5.3 Hz, 2H, NCH_2), 4.32 (t, J = 5.3 Hz, 2H, OCH_2), 6.72 (t, J = 1.0 Hz, 2H, CH_{vinyl}). $^{13}C\{^1H\}$ NMR (100.59 MHz, $CDCl_3$, 298 K): δ = 30.64 (2C, CH_3), 36.60 (2C, NCH_2), 55.54 (1C, $C(CH_3)_2Br$), 62.93 (OCH_2), 134.31 (2C, CH_{vinyl}), 170.37 (1C, CO_{ester}), 171.41 (2C, CO_{imide}). Anal. Calcd for $C_6H_7NO_3$: C, 41.40; H, 4.17; N, 4.83; Br, 27.54. Found: C, 41.62; H, 4.27; N, 4.48; Br, 27.43. Mass spectrometry (+EI) *m*/*z* (%): 292 [MH^+] (3), 290 [MH^+] (3), 219 (5), 210 (14), 151 (7), 149 (7), 140 (17), 124 (80), 123 (100), 121 (40), 110 (63), 86 (27), 84 (42), 82 (36), 70 (68), 69 (90).

Polymerization. General Procedure: The *N*-(*n*-alkyl)-2-pyridylmethanimine ligand, an appropriate initiator, and the monomer were charged to a dry Schlenk tube along with toluene or anisole as the solvent. The tube was sealed with a rubber septum and subjected to five freeze−pump−thaw cycles. This solution was then cannulated under nitrogen into another Schlenk tube, previously evacuated and filled with nitrogen, containing Cu(I)Br and a magnetic follower. The brown solution was subsequently heated to the desired temperature with constant stirring (t = 0). Samples were removed periodically using a degassed syringe for molecular weight and conversion analysis. At the end of the polymerization, the mixture was diluted with 50 mL of toluene, and air was bubbled for at least 12 h. After filtration through a Celite pad and purification on a short neutral alumina column, the solvent was removed under reduced pressure to give the polymers as yellow-brown oils. The purification processes vary between the different polymers and will be specified case by case.

Polymers 6. Purification Procedure: Extensive dialysis in water (Millipore, regenerated cellulose, MWCO 1 kDa, filtration area of 0.23 m^2) was followed by freeze-drying to give the products **6** as pale-yellow viscous oils. **6a:** $m \approx 9$; n = 28; M_n (NMR) = 13.6 kDa; M_w/M_n (GPC) = 1.11; conversion = 100%; initiating efficiency = 100%; ligand *N*-(ethyl)-2-pyridylmethanimine; ligand/Cu(I)Br/$[initiator]_0$/$[monomer]_0$ = 2:1:1:28; solvent/monomer = 1:1 (v/v); T = 40 °C. **6b:** $m \approx 9$; n = 8; M_n (NMR) = 4.1 kDa; M_w/M_n (GPC) = 1.08; conversion = 100%; initiating efficiency = 75%; ligand *N*-(ethyl)-2-pyridylmethanimine; ligand/Cu(I)Br/$[initiator]_0$/$[monomer]_0$ = 2:1:1:6; solvent/monomer = 1:1 (v/v); T = 40 °C.

Polymers 9. Purification Procedure: Polymer was precipitated into a 1:1 mixture of Et_2O/petroleum ether to give the polymers **9** as pale-yellow viscous oils. **9a:** $m \approx 9$; n = 69; M_n (NMR) = 33.0 kDa; M_w/M_n (GPC) = 1.19; conversion = 53%; initiating efficiency = 61%; ligand *N*-(propyl)-2-pyridylmethanimine; [ligand]/[Cu(I)Br]/$[initiator]_0$/$[monomer]_0$ = 5:2.5:1:80; solvent/monomer = 2:1 (v/v); T = 27 °C. **9b:** $m \approx 9$; n = 17; M_n (NMR) = 8.4 kDa; M_w/M_n (GPC) = 1.08; conversion = 37%; initiating efficiency = 44%; ligand *N*-(propyl)-2-pyridylmethanimine; [ligand]/[Cu(I)Br]/$[initiator]_0$/$[monomer]_0$ = 1.6:0.8:1:20; solvent/monomer = 2:1 (v/v); T = 21 °C.

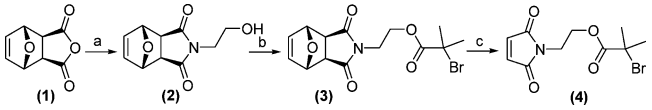
Polymers 11. Purification Procedure: The solution obtained after the alumina column (volume ≈ 120 mL) was added dropwise, under stirring, to 1.7 L of petroleum ether (dropping time ca. 30 min) in order to precipitate the polymer. After filtration, the off-white solid was dissolved in the minimum amount of CH_2Cl_2 and passed through a short neutral alumina column, eluting with the same solvent. After filtration, the polymers **11** were obtained as white solids. **11a:** n = 170; M_n (NMR) = 34.5 kDa; M_w/M_n (GPC) = 1.15; conversion = 68%; initiating efficiency = 71%; ligand *N*-(propyl)-2-pyridylmethanimine; [ligand]/[Cu(I)Br]/$[initiator]_0$/$[monomer]_0$ = 12:6:1:180; solvent/monomer = 2:1 (v/v); T = 28 °C. **11b:** n = 31; M_n (NMR) = 6.6 kDa; M_w/M_n (GPC) = 1.28; conversion = 63%; initiating efficiency = 63%; ligand *N*-(propyl)-2-pyridylmethanimine; [ligand]/[Cu(I)Br]/$[initiator]_0$/$[monomer]_0$ = 2:1:1:30, ligand/Cu(I)Br/$[initiator]_0$/$[monomer]_0$ = 2:1:1:30; solvent/monomer = 2:1 (v/v); T = 21 °C.

Retro-Diels−Alder Reactions. General Procedure: Synthesis of 10b. A solution of **9b** (3.0 g, 0.36 mmol) in toluene (25 mL) was warmed to reflux, and the reaction was monitored by ^1H NMR analysis with samples taken at regular intervals. After 7 h, the solvent was removed under reduced pressure to give the polymer **10b** as a pale-orange oil. M_n (NMR) = 9.0 kDa; M_w/M_n = 1.09.

Conjugation with Reduced Glutathione. General Procedure: Polymer 13b. Product **13b** (12 mg, estimated content of H_2O = 20% (w/w), 1.6 μmol) was dissolved in 0.8 mL of 100 mM phosphate buffer in D_2O (pH 6.5) containing *p*-toluenesulfonic acid sodium salt (0.27 mg, 1.9 mmol (1 mg dissolved in 3.0 mL of buffer in D_2O, then 0.8 mL of this solution employed for dissolving **13b**)) as the ^1H NMR standard. This solution was then titrated by successive addition of 10 μL aliquots of a 16 mM solution of reduced glutathione in 100 mM phosphate buffer in D_2O (pH 6.5). ^1H NMR spectra were recorded 10 min after each addition. **10b:** The same conditions described above for polymer **13b** were employed, except that 0.2 equiv of aliquots was added. NOTE: both **13b** and **10b** are stable in 100 mM PBS buffer in D_2O, pH 6.5, for at least 24 h.

Conjugation with BSA: All of the solutions employed for these experiments were carefully degassed by gently bubbling nitrogen for at least 30 min. In the case of BSA, the desired amount of oxygen-free water was transferred via a degassed syringe into a vial equipped with a rubber septum, containing BSA stored under a nitrogen atmosphere. **General Procedure: Polymer 13a.** Total volume: 1.0 mL. Solution **a**: 0.5 mL of 0.10 mM BSA in water. Solution **b**: 0.2 mL of 500 mM potassium phosphate buffer, pH 7.4. Solution **c**: 0.3 mL of 3.3 mM polymer solution in water. Solutions **a**, **b**, and **c** were transferred via a degassed syringe into a 2 mL vial equipped with a rubber septum that was previously flushed with nitrogen. After 18 h, several aliquots were taken for SDS−PAGE and FPLC analysis.

Scheme 1[a]



[a] Reagent and conditions: (a) ethanolamine, MeOH, 0 °C to reflux, 4 h, 42%; (b) 2-bromo isobutyryl bromide, Et_3N, 0−25 °C, 93%; (c) toluene reflux, 8 h, quant.

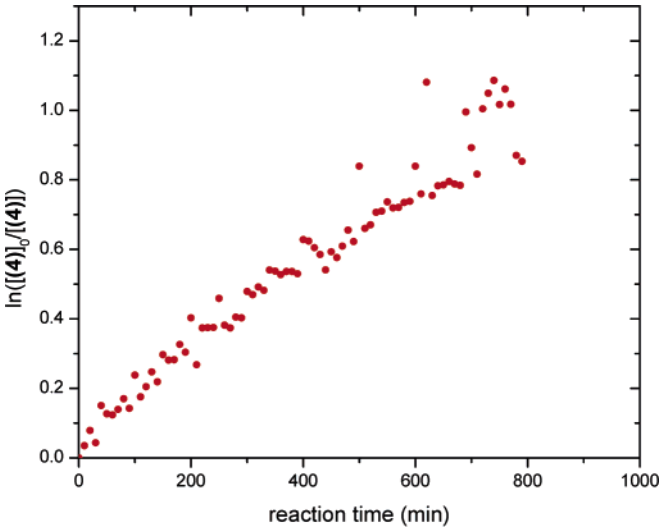


Figure 1. ^{1}H NMR online polymerization of methoxyPEG$_{(475)}$ methacrylate using **4** as the initiator in toluene-d_8. The reaction was monitored by following the decrease of the maleimide vinyl signal with time. [ligand]/[CuBr]/[monomer]/[**4**] = 2:1:10:1; T = 30 °C.

Results and Discussion

α-Functional polymers containing unprotected functionality, such as hydroxyl, amido, and tertiary amine, can be easily obtained by TMM-LRP starting from appropriate initiators.[20,21] In our case, however, this simple strategy is problematic as the maleimido initiator is itself a polymerizable monomer, and therefore, copolymerization into the growing chain occurs.[22] To verify this, we prepared the initiator **4** following the synthetic protocol shown in Scheme 1.[23,24]

Polymerization of monomethoxyPEG$_{(475)}$ methacrylate was carried out in an NMR tube within the cavity of the NMR spectrometer in the presence of Cu(I)Br, and an iminopyridine ligand[25] with **4** as the initiator showed the maleimide initiator acts, indeed, as a co-monomer and is incorporated into the polymer backbone following good first-order kinetics (Figure 1).

Thus, the maleimide moiety needs to be introduced via an indirect approach into the polymers following two independent approaches: (a) a post-functionalization of a preformed primary amine-terminated hydrophilic polymer, or (b) the use of a "protected" maleimide initiator for the polymerization step followed by deprotection to give the expected α-functional polymers (Scheme 2).

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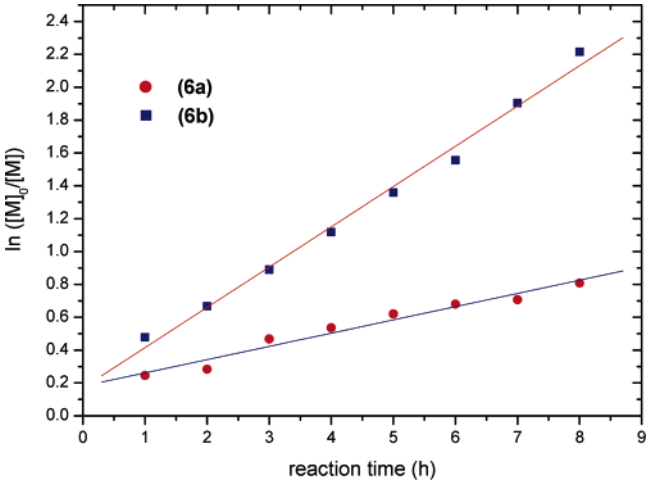


Figure 2. First-order kinetic plot for the synthesis of NH Boc-terminated polymers **6**. Reaction conditions: **(6a)**, [Cu(I)Br]/[ligand]/[initiator]$_0$/[monomer]$_0$ = 1:2:1:28; **(6b)**, [Cu(I)Br]/[ligand]/[initiator]$_0$/[monomer]$_0$ = 1:2:1:6; T = 40 °C.

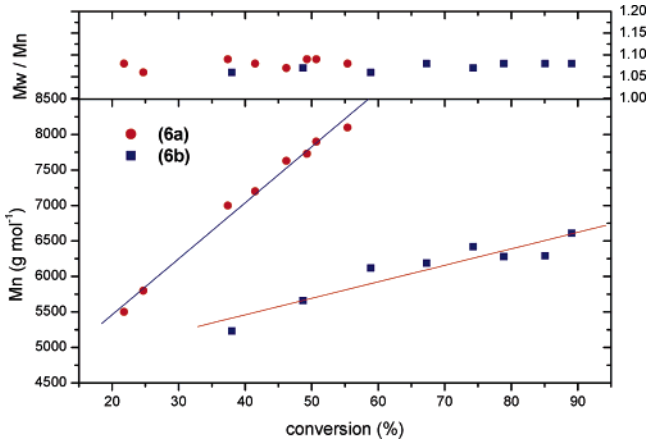
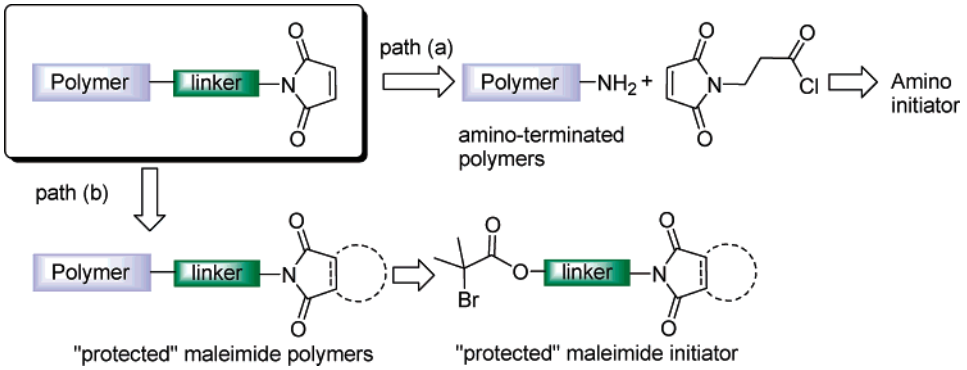


Figure 3. Evolution of both the M_n (SEC) and M_w/M_n with conversion for polymers **6**.

Path a: Post-Functionalization Protocol. To obtain the α-primary amino-terminated polymers, methodology involving a Boc-protected amino initiator was developed. Polymerization of monomethoxyPEG$_{(475)}$ methacrylate in the presence of Cu(I)Br and an iminopyridine ligand in the presence of the protected amino initiator **5**, obtained in a one-pot two-step reaction from 3-amino-1-propanol, showed a linear dependence on the monomer concentration and gave the expected polymers **6**, with excellent control over the molecular weight, and polymers with narrow MWD in all cases (Scheme 3, Figures 2 and 3). It is noted that the plots do not pass through the origin which is ascribed to changes in the catalyst in the early stages of the reaction leading to an observed change in rate. There can also be a higher concentration of free radicals formed, which in tandem with the higher rate of diffusion of the low mass oligomers can lead to increases in radical−radical termination reactions. In all copper-mediated living radical polymerizations, there is an equilibrium state between Cu(I) and Cu(II) species which needs to be established over approximately the first 10% monomer conversion.

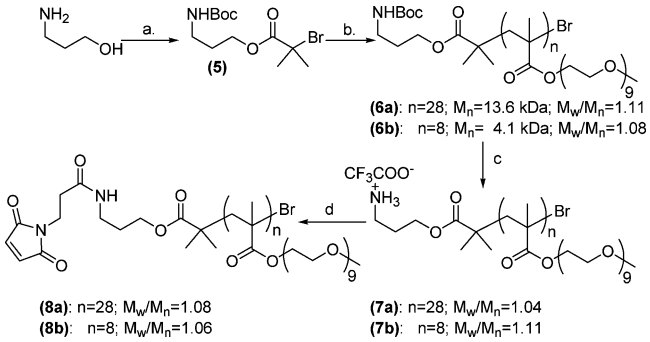
Polymers **6** were subsequently deprotected under acidic conditions to the corresponding α-functional amino-terminated trifluoroacetate salts **7** which, in the presence of DIPEA and an

Scheme 2. Two Retrosynthetic Approaches to α-Maleimide-Functional Macromolecules[a]



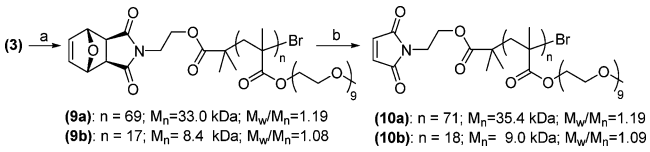
[a] Path a via a post-functionalization protocol on amino-terminated polymers; path b via protected maleimide polymers.

Scheme 3 [a]



[a] Reagents and conditions: (a) *i*. Boc_2O, THF, rt; *ii*. 2-bromo isobutyryl bromide, Et_3N, 0 °C; (b) Cu(I)Br/*N*-(ethyl)-2-pyridylmethanimine/methoxyPEG$_{(475)}$ methacrylate, toluene (50% v/v), 40 °C; (c) CF_3COOH, CH_2Cl_2, rt; (d) 3-maleimidopropionyl chloride, DIPEA, CH_2Cl_2, 0 °C to rt.

Scheme 4[a]



[a] (a) Cu(I)Br/*N*-(*n*-propyl)-2-pyridylmethanimine/methoxyPEG$_{(475)}$ methacrylate, toluene, 27 °C; (b) toluene reflux, 7 h.

excess of 3-maleimidopropionyl chloride, furnished the maleoyl−polymers **8** with an 80−85% content in reactive terminal groups, values comparable to those typical for the commercially available linear PEG−maleimide derivatives.

Path b: Protected Maleimido Polymers. We reasoned that the problem of the incorporation of the maleimide moiety into the polymer backbone could have been circumvented by using the intermediate **3** as the *actual* initiator followed by reintroduction of the desired reactive chain-end by a retro-Diels−Alder reaction by refluxing the resulting protected polymer intermediates in toluene (Scheme 4). This approach seemed to be very promising, due to the virtually 100% yields observed for the retro-Diels−Alder deprotection of **3** to give the initiator **4**. Polymerization of methoxyPEG$_{(475)}$ methacrylate in the presence of **3** and Cu(I)Br/pyridinylimine ligand as the catalytic system followed refluxing toluene afforded the expected maleimido polymers **10** with a range of controlled molecular weights and narrow molecular weight distribution. First-order kinetics in monomer and a linear evolution of the molecular weights were observed (Figures 4 and 5); again, we see changes in the rate at the start of the reaction.

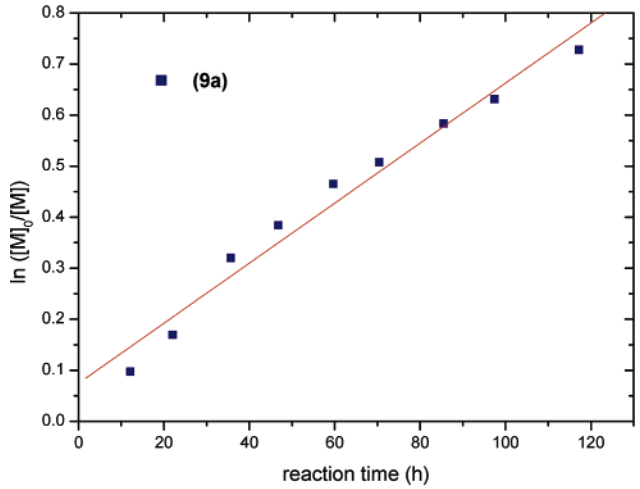


Figure 4. Typical first-order kinetic plot reported for **9a**. Reaction conditions: [Cu(I)Br]/[ligand]/[initiator]$_0$/[monomer]$_0$ = 2.5:5:1:80; T = 27 °C.

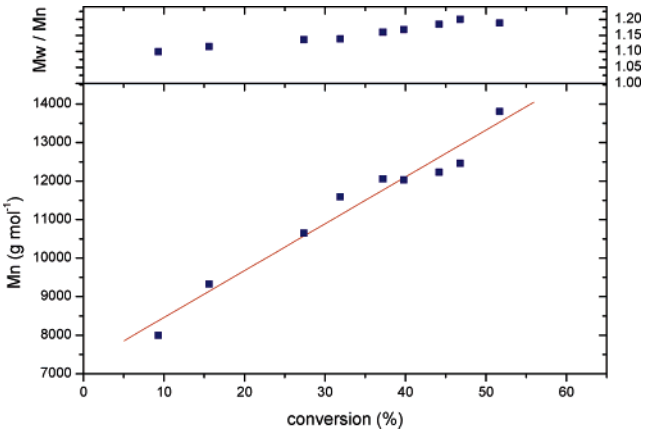
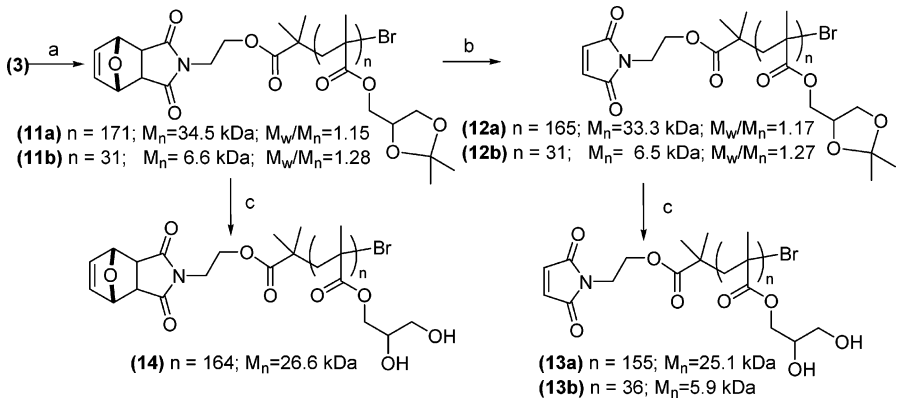


Figure 5. Evolution of both the M_n (SEC) and M_w/M_n with conversion for polymer **9a**.

The retro-Diels−Alder reaction of polymers **9** occurred with virtually 100% efficiency, which is in line with what was observed for the synthesis of initiator **4**. As expected, the deprotection step, monitored by ^{1}H NMR analysis following the decreasing of the oxatricylo vinyl signals and the consequent increasing of the maleimide peak, gave a first-order kinetic plot (Figure 6). In theory, the double bond of the initiator could copolymerize to give branched polymers. Indeed, copolymerization of nonacrylic monomers, such as ethylene and α-olefins, with acrylate monomers under ATRP conditions has been

Scheme 5[a]



[a] (a) Cu(I)Br/*N*-(*n*-propyl)-2-pyridylmethanimine/(2,2-dimethyl-1,3-dioxolan-4-yl)methyl methacrylate, anisole, 27 °C; (b) toluene reflux, 7 h; (c) dioxane/1 N HCl, 0−25 °C, 24 h.

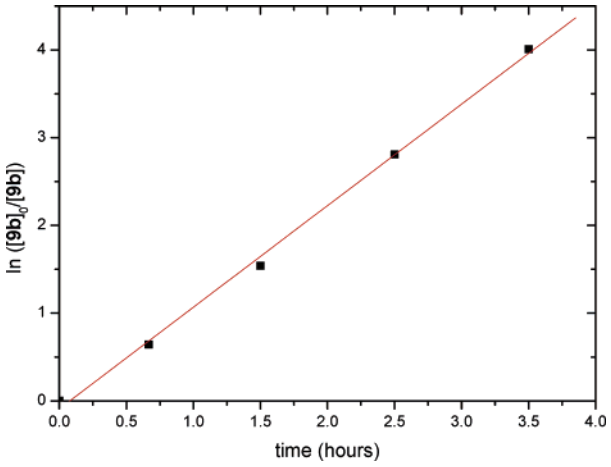


Figure 6. First-order kinetic plot for the retro-Diels−Alder reaction on polymer **9b**; line is regression fit through data points.

reported by Sen and co-workers.[22] However, ^{1}H NMR analysis using mesitylene as the internal standard showed that the integrals of the vinylic protons of the initiator did not change significantly during the polymerization reaction, and therefore that under our experimental conditions, the initiator was not incorporated into the polymer backbone. The conversions were kept below 60% in order to avoid undesired copolymerization of the initiator/α-functional polymer.

Thus, both the synthetic strategies led to the desired α-functional maleimido polymers in very good yields and purity. However, we identified the approach via the protected maleimido polymers (path b) as the most convenient, as the purification processes of the polymer intermediates are simple and the final polymers are *all* end-functionalized with the maleimido moiety. It is known that both the shape and the size of the polymer employed for protein functionalization can influence the biological properties of the conjugates. Bearing in mind these simple concepts and having identified among the ones studied in the present work the best method for the synthesis of maleimide-terminated polymers, we decided to prepare a different type of α-functional water-soluble compound bearing shorter side-chains. In particular, we turned our attention toward poly(glycerol)monomethacrylates, a class of polymers widely employed for the preparation of biocompatible materials.[26,27]

The synthetic protocol developed is similar to the one proposed by Nakahama and co-workers,[28] with modifications arising from the introduction of the maleimide unit into the polymer backbone. Polymerization of (2,2-dimethyl-1,3-dioxolan-4-yl)methyl methacrylate in the presence of **3** as the initiator afforded polymers **11** that were first subjected to a retro-Diels−Alder reaction and then to a ketal deprotection under acidic conditions to give the expected maleimide-terminated poly(glycerol)methacrylates **13** (Scheme 5), again with narrow molecular weight distributions.

Conjugation with Model Peptide and Protein. These polymers were assessed for their reactivity toward a model thiol-functionalized peptide, reduced glutathione, and protein, bovine serum albumin (BSA). Solutions of polymers **10b** and **13b** (100 mM phosphate buffer in D_2O, pH 6.5) were titrated at ambient temperature with reduced glutathione, a tripeptide (γ-Glu-Cys-Gly) containing one sulfydryl unit, and the decrease of the maleimide vinyl signal was followed by ^{1}H NMR (Figure 7).

The excellent linear conversion observed outlines the high selectivity, under the experimental conditions employed, of the maleimide polymers toward thiols, as the amino group present in the glutathione structure did not react with the reactive polymer chain-end. These results show that the polymer reacts very quickly (less than 10 min for polymer **13b**) with the tripeptide, and that the quantification of the maleimide content in the polymers via ^{1}H NMR was accurate. It is important to point out that along with the disappearance of the maleimide vinyl signal, the increasing of some peaks related to the peptidic part of the conjugate was observed (Figure 8). Those peaks were found to be analogous to those of a model compound prepared by reaction of glutathione with *N*-methyl maleimide (see Supporting Information).

Bovine serum albumin (BSA), a commercially available protein, was chosen as a model substrate for the protein−polymer conjugation reactions. The latter were carried out in aqueous solutions at pH 7.4 using various concentrations of maleimide polymers, under oxygen-free conditions, to prevent the aggregation of the protein through thiol−disulfide inter-

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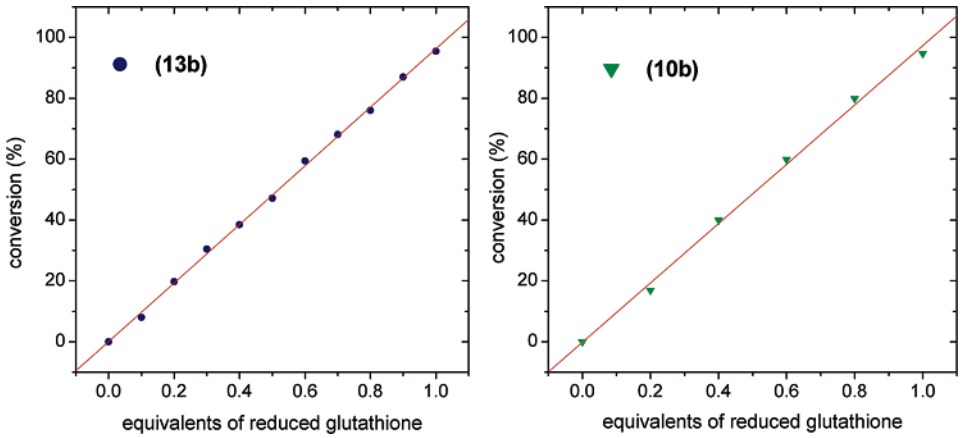

Figure 7. Titration of **13b** (left) and **10b** (right) with reduced glutathione (100 mM PBS in D_2O, pH 6.5); the reaction was monitored by ^1H NMR analysis.

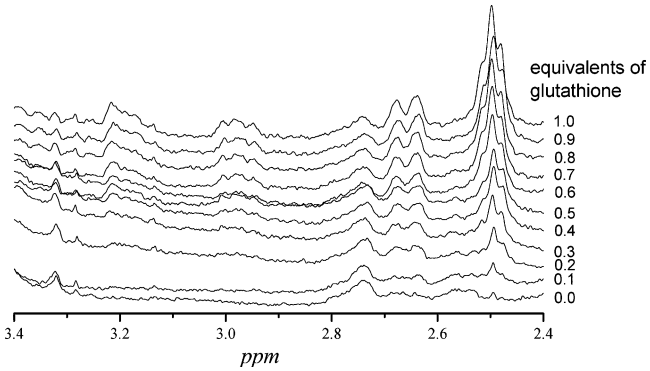

Figure 8. Titration of **13b** with reduced glutathione: partial ^1H NMR spectrum. The intensity of the signals relative to the peptidic part of the conjugate increased with the amount of added reduced glutathione increasing.

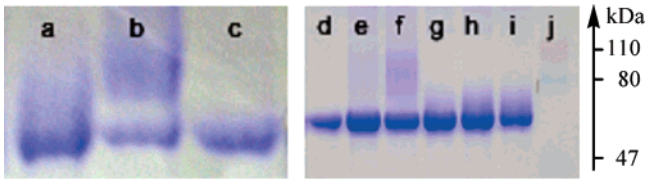

Figure 9. SDS−PAGE for the conjugation reaction of BSA with (a) 20 equiv of **13b**; (b) 20 equiv of **13a**; (c) 20 equiv of **14**; (d) 0.5, (e) 1.5, and (f) 15 equiv of **10a**; (g) 0.5, (h) 1.5, and (i) 15 equiv of **10b**; (j) molecular weight markers.

change reactions[29] or via oxidative formation of disulfide bridges.

SDS−PAGE analysis (Figure 9) of some conjugates obtained using the polymers **10** and **13** showed the presence of bands at higher molecular weight than that with BSA in each case (66.5 kDa). The molecular weights of these new bands were found to be dependent on the molecular weight of the polymer used, and for the polymers **10**, the band intensities were more pronounced after the conjugation with a larger excess of the maleimide-terminated polymer. No conjugates were observed when protected maleimide-terminated polymers **9** and **14** were employed, which allowed us to exclude the presence of adducts formed through noncovalent interactions between the protein and the polymers. An FPLC analysis of the reaction mixture obtained using **13a** confirmed the presence of the protein−conjugate adduct (Figure 10).

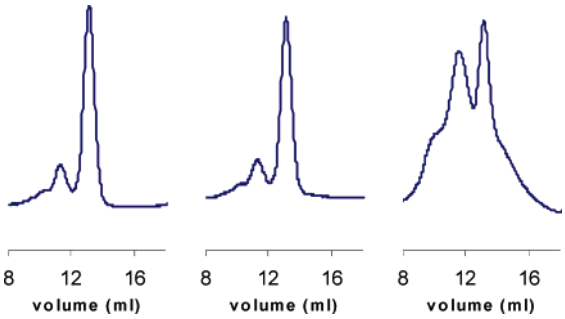

Figure 10. FPLC analysis of (a) BSA, (b) BSA + **14**, and (c) BSA + **13a**.

Titration of the commercially available BSA showed that approximately 55% of the BSA molecules actually contain a sulfydryl unit available for the conjugation reaction, which is in line with previous reports.[30] This result, in part, explains why in the SDS−PAGE and FPLC analyses of the conjugation mixtures, some unreacted BSA is always detected.

Conclusions

In summary, we have reported the synthesis of new hydrophilic water-soluble maleimide functional polymers obtained via copper-catalyzed living radical polymerization (LRP). Two independent synthetic pathways have been successfully employed for the preparation maleimide-terminated water-soluble polymeric bioconjugates. Moreover, a novel methodology for the synthesis of amino-terminated polymer intermediates has been presented. Pure α-monofunctionalized macromolecules have been prepared, which avoids the potential for cross-linking side-reactions that sometimes are observed in PEGylation chemistry, arising from bifunctional PEG chains present as impurities. These α-functional methacrylate polymers have been successfully employed in coupling reactions with both a tripeptide (reduced glutathione) and a model protein (BSA) for the synthesis of a series of conjugates. Preliminary results indicate that the yields vary to a certain extent with the polymer structure, presumably due to steric hindrance. Further experiments are aimed at investigating the different biohybrids. The synthetic approach is very general and is applicable to many (poly)peptide thera-

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peutics and enzymes and to a wider range of monomers that can be polymerized under TMM-LRP conditions. We believe these results are particularly intriguing as they provide a new powerful tool in the development of (poly)peptide conjugates that might complement the technologies currently employed in this field.

Acknowledgment. The authors thank the Marie Curie Fellowship Network (G.M., MEIF-CT-2003-501305), the University of Warwick (F.L. and L.T.) for funding this work, and Drs. A. Jarvis, A. J. Carmichael, and A. G. Steward of Warwick Effect Polymers and Dr. J. L. Irwin for helpful discussions. Professor A. E. Rowan and Professor R. J. M. Nolte are acknowledged for their support of the biohybridization project in Nijmegen.

Supporting Information Available: Experimental section, including the full characterization of initiators, maleimide polymers, and all the intermediates, the synthesis of **5**, **7**, **8**, and **12**−**14**, and the conjugation reactions procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

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