Biocompatible, Thermoresponsive, and Biodegradable: Simple Preparation of "All-in-One" Biorelevant Polymers

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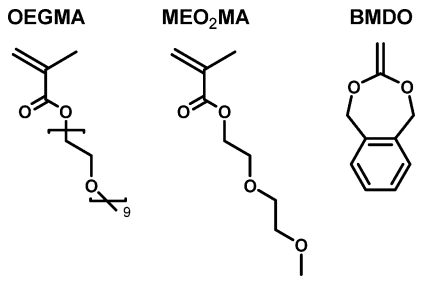
Introduction. Synthetic polymer materials have found widespread applications in almost all areas of modern biosciences (e.g., delivery, diagnostics, implants, bioseparation, and microarrays).[1] However, surprisingly, only a handful of established polymers are widely used in pharmaceutical and clinical products nowadays. For instance, FDA-approved polymers such as poly(ethylene glycol) (PEG) or poly(lactide-*co*-glycolide) (PLGA) have been successfully exploited in numerous commercial bioapplications within the past few decades. Still, the advanced biotechnology applications of the 21st century certainly require "smarter" biomaterials with more sophisticated properties. In that regard, polymer chemists have an important role to play and should offer new opportunities and insights for designing the biomaterials of the future.

We[2−5] and others[6,7] recently emphasized that nonlinear PEG analogues, constructed from short oligo(ethylene glycol) macromonomers, are highly relevant macromolecules for bioapplications. First, these PEG-based polymers may be synthesized using straightforward controlled radical polymerization techniques such as atom transfer radical polymerization (ATRP) or reversible addition−fragmentation transfer polymerization (RAFT) but also functionalized using versatile ligation tools such as "click" chemistry.[8] Thus, they can be easily connected to a wide variety of materials, including planar inorganic substrates (e.g., gold or glass surfaces), solid or soft-matter nanoparticles (e.g., nanocarriers, contrast agents), or biological structures (e.g., proteins). Moreover, nonlinear PEG analogues can exhibit stimuli-responsive properties, which are typically not attainable with linear PEG.[2,4,9] For example, we recently reported that the atom transfer radical copolymerization of two oligo(ethylene glycol) methacrylates of different chain lengths, namely 2-(2-methoxyethoxy)ethyl methacrylate (MEO_2MA, Scheme 1) and oligo(ethylene glycol) methacrylate (OEGMA, Scheme 1), leads to the formation of thermoresponsive copolymers with a precisely tunable lower critical solution temperature (LCST) in water.[2,4] The phase transitions measured for the copolymers P(MEO_2MA-*co*-OEGMA) were found to be reversible and relatively insensitive to important parameters such as concentration of the copolymer in water, ionic strength and chain length.[2] Hence, copolymers P(MEO_2MA-*co*-OEGMA) appear as promising alternative to conventional poly(*N*-isopropylacrylamide) (PNIPAM) for bioapplications and more generally for building any kind of thermoresponsive materials. For example, some recent reports describe the synthesis of thermoresponsive P(MEO_2MA-*co*-OEGMA)-based microgels and surface brushes.[7,10] Overall, these novel "smart" polymers are expected to exhibit a fairly high degree of biocompatibility as they are principally composed of biocompatible oligo(ethylene glycol) segments. Several studies already indicated that materials based on oligo(ethylene glycol) macromonomers exhibit an excellent in vitro or in vivo biocompatibility.[5,6,11] As an additional example, Figure S1 (Supporting Information) illustrates that copolymers P(MEO_2MA-*co*-OEGMA) are not cytotoxic.

Scheme 1. Molecular Structures of the Three Comonomers Used in the Present Study



Yet, one potential limitation of P(MEO_2MA-*co*-OEGMA) copolymers is indeed the nondegradability of their carbon−carbon backbone. This aspect could hamper the widespread adoption of these macromolecules in the biomedical field, in particular for in vivo applications. The goal of the present communication is to demonstrate that it is possible to introduce labile linkages in the backbone of these copolymers, while retaining stimuli-responsive and biocompatible properties. Various types of labile moieties may be exploited for preparing biodegradable materials, for example anhydrides, disulfides, or esters.[12] The latter option was selected in the present work. During the 1980s and 1990s, important developments have been made in the field of free radical ring-opening polymerization.[13−15] For instance, Bailey and co-workers reported that cyclic ketene acetals polymerize via a radical ring-opening mechanism and lead to the formation of main-chain polyesters.[13] Interestingly, it was reported that some of these monomers copolymerize with methyl methacrylate or styrene. Yet, some of these copolymerization data have been a subject of debate. For instance, the reactivity ratios measured for copolymerizations involving 2-methylene 1,3-dioxepane (MDO) reflect a low copolymerization tendency.[15] Nevertheless, Agarwal and co-workers recently evidenced that 5,6-benzo-2-methylene-1,3-dioxepane (BMDO, Scheme 1) could be successfully used as a comonomer to prepare degradable polystyrene, poly(methyl methacrylate), and PNIPAM.[16−18]

The bulk atom transfer radical terpolymerization of MEO_2-MA, OEGMA and BMDO was studied at 90 °C and in the presence of the homogeneous ATRP catalytic system copper-(I) chloride/2−2′ bipyridyl. In order to design a complete series of thermoresponsive copolymers, various comonomer compositions were screened (Table 1). In all cases, the terpolymers could be obtained in high yields within a few hours. SEC measurements in tetrahydrofuran (THF) indicated that the formed polymers are relatively well-defined. In addition, ^{1}H NMR analysis of the raw polymerization mixtures confirmed that the

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Table 1. Properties of the Terpolymers P(MEO_2MA-*co*-OEGMA-*co*-BMDO) Prepared Using ATRP[a]

	MEO_2MA/OEGMA/BMDO	$convn_{MA}$[b]	$convn_{BMDO}$[c]	F_{esters}[c] (%)	$F_{defects}$[c] (%)	M_n[d]	M_w/M_n[d]	LCST[e] (°C)
1	40:40:20	0.94	0.40	6.2	1.0	15 400	1.32	67
2	50:30:20	0.95	0.46	6.7	1.7	14 700	1.35	59
3	55:25:20	0.95	0.58	5.6	1.1	15 400	1.45	54
4	60:20:20	0.95	0.46	7.0	1.9	12 700	1.38	47
5	65:15:20	0.97	0.57	6.3	1.3	14 000	1.60	41
6	70:10:20	0.97	0.53	7.9	2.2	12 200	1.65	31

[a] Experimental conditions: 5 h, bulk, 90 °C, $[MEO_2-MA]_0 + [OEGMA]_0 + [BMDO]_0)/[MBP]_0/[CuCl]_0/[Bipy]_0 = 100/1/1/2$. [b] Overall monomer conversion measured by ^1H NMR for both methacrylates. [c] Calculated by ^1H NMR. [d] Measured by SEC in THF. [e] Measured by turbidimetry for aqueous solutions with a concentration of 3 mg·mL^{-1}; the presented values are the inflection points of the heating cycles.

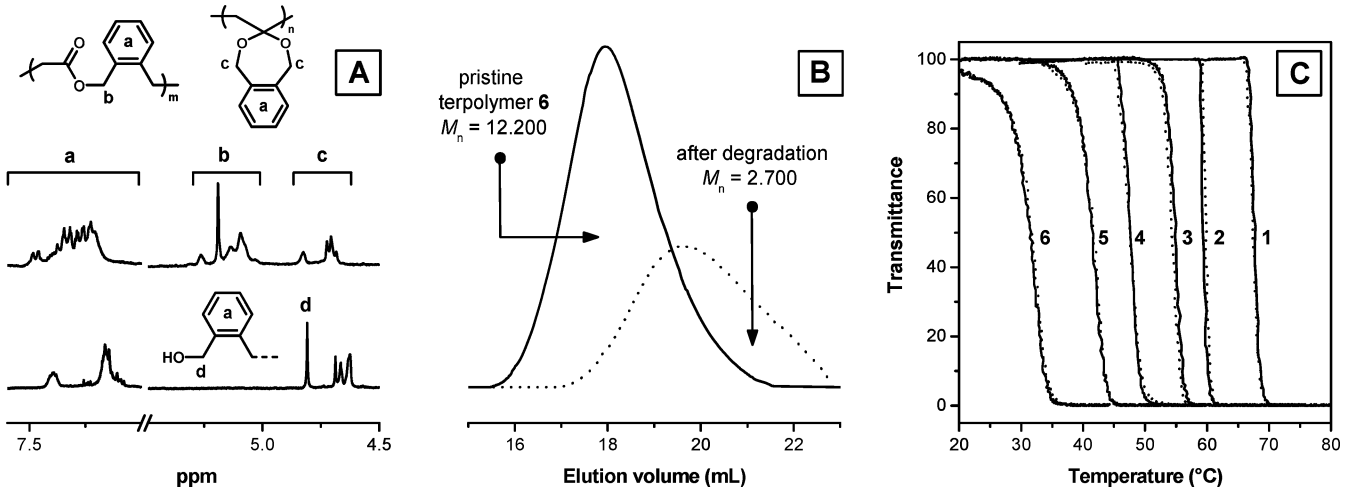

Figure 1. (A) ^1H NMR recorded at room temperature in acetone-d_6 for terpolymer **6** (Table 1, entry 6) after synthesis and purification (top) and after chemical degradation in the presence of KOH (bottom). (B) SEC chromatograms measured for terpolymer **6** before (full line) and after (dotted line) chemical degradation. (C) Plots of transmittance as a function of temperature (670 nm, 1 °C·mn^{-1}) measured for aqueous solutions (3 mg·mL^{-1}) of terpolymers P(MEO_2MA-*co*-OEGMA-*co*-BMDO) of various composition (Table 1, entries 1−6). Solid lines: heating cycles. Dotted lines: cooling cycles.

overall conversion of MEO_2MA and OEGMA was almost quantitative in all cases. The conversion of BMDO was found to be in the range 40−60%, which is in good agreement with previous copolymerization studies with methyl methacrylate.[17] ^1H NMR spectra recorded for the purified polymers indicate that BMDO polymerized mainly via a ring opening mechanism (Figure 1A). Indeed, a broad signal corresponding to the methylene protons of the main-chain benzyl esters was observed in all spectra at 5.0−5.3 ppm.[16,17] However, another region, probably corresponding to nonopened cyclic acetals (i.e., units incorporated via a vinyl addition mechanism), could be observed at 4.65−4.85 ppm.[16,17] The integration of both signals allowed calculation of the molar fraction of main-chain esters F_{esters} and the molar fraction of defects $F_{defects}$ in the formed copolymer (Table 1). Generally speaking, the polymers contain 5−7 mol % of main-chain esters and approximately 1−2 mol % of defects.

The incorporation of hydrophobic BMDO units in the backbone of the copolymers did not affect notably their thermoresponsive and biocompatible properties. Figure 1C shows the phase transitions observed by turbidimetry for the various copolymers listed in Table 1. In all cases, narrow and reversible phase transitions were measured, indicating that the terpolymers have a homogeneous chain-to-chain composition.[2] Furthermore, as previously reported, the observed LCST values closely depend on the molar fraction of OEGMA in the initial comonomer feed.[4] In addition, Figure 2 indicates that the formed terpolymers exhibit, similarly to standard linear poly(ethylene glycol), a very low in vitro cytotoxicity.

The hydrolytic degradation of the copolymers P(MEO_2MA-*co*-OEGMA-*co*-BMDO) was studied by ^1H NMR and SEC. The polymers were dissolved in a KOH solution and stirred at room

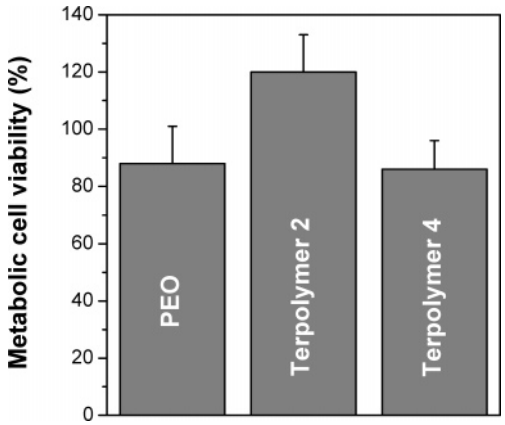

Figure 2. Metabolic cell viability measured for human hepatocellular carcinoma (HepG2) cell lines incubated at 37 °C in the presence of either a linear poly(ethylene oxide) (PEO, M_n = 20 000 g·mol^{-1}), terpolymer **2** (Table 1, Entry 2), or terpolymer **4** (Table 1, Entry 4).

temperature for 1 day. Figure 1B compares, for example, the SEC chromatograms recorded for copolymer **6** (Table 1, Entry 6) before and after hydrolysis. The average molecular weight of the copolymer decreased by a factor of 4 after KOH treatment, thus confirming that (i) the hydrolysis of the labile main-chain esters occurred successfully and (ii) the BMDO units are distributed rather regularly along the copolymer backbone (i.e., no pronounced gradient effect). Furthermore, ^1H NMR confirmed main-chain degradation. As shown in Figure 1A, the signal of the main-chain benzyl esters at 5.0−5.3 ppm completely disappeared after hydrolytic treatment and was replaced by a new peak at 4.8 ppm. The latter corresponds most probably to the methylene protons of the benzyl alcohol chain-ends

obtained after hydrolytic cleavage. Interestingly, ^{1}H NMR also evidenced that the side-chain esters of MEO_2MA and OEGMA were not noticeably hydrolyzed under the studied conditions. The signal of the methylene protons neighboring the methacrylate esters (3.95−4.35 ppm) was almost identical before and after basic treatment (data not shown).

Additionally, the enzymatic degradation of P(MEO_2MA-*co*-OEGMA-*co*-BMDO) was studied. The copolymers were incubated at 37 °C in the presence of immobilized *Candida antarctica* lipases. After 24 h, ^{1}H NMR and SEC indicated that even though the copolymers were not entirely degraded (i.e., around 40% of the main-chain esters were hydrolyzed), the enzymatic solutions led to higher level of degradation than blank solutions. Thus, the lipases are obviously able to catalyze the hydrolysis of the polymer backbones. However, in the studied conditions, the degradation is perhaps not exclusively due to an enzyme mechanism but most probably to a combination of hydrolytic and enzymatic chain cleavage.

In conclusion, "smart" PEG-based materials could be prepared via a simple one pot atom transfer radical terpolymerization. These novel macromolecules exhibit a sharp LCST in aqueous solution, have a very low cytotoxicity and moreover can be hydrolytically degraded into short oligomers. Hence, these novel materials could be of particular utility for biorelated technologies and applications.

Experimental Part. Chemicals. 2-(2-Methoxyethoxy)ethyl methacrylate (Aldrich, 95%), oligo(ethylene glycol) methyl ether methacrylate (Aldrich, $M_n = 475$ g·mol^{-1}), methyl 2-bromopropionate (MBP) (Aldrich, 98%) and 2,2′ bipyridyl (Bipy) (Fluka, 98%) were used as received. Lipase immobilized from *C. antarctica* (2.0 U/mg) and lipase A *C. antarctica* CLEA (1.7 U/mg) were purchased from Fluka biochemika. Copper(I) chloride (Acros, 95%) was washed with glacial acetic acid in order to remove any soluble oxidized species, filtered, washed with ethanol and dried. 5,6-Benzo-2-methylene-1,3-dioxepane (BMDO) was synthesized as previously reported.[16,17] To prevent degradation, BMDO should be stored under dry argon.

Example of Bulk Atom Transfer Radical Terpolymerization of MEO_2MA, OEGMA, and BMDO. Copper chloride, 2,2′-bipyridyl, and BMDO were added to a Schlenk tube sealed with a septum. The tube was purged with dry argon for a few minutes. Then, a degassed mixture of 2-(2-methoxyethoxy)-ethyl methacrylate and oligo(ethylene glycol) methyl ether methacrylate was added through the septum using a degassed syringe. Last, methyl 2-bromopropionate was added with a microliter syringe. The mixture was heated at 90 °C in an oil bath for 5 h. The experiment was stopped by opening the flask and exposing the catalyst to air. The final mixture was diluted with ethanol and purified by dialysis against a water/ethanol mixture 1:1 v/v (Roth, ZelluTrans membrane, molecular weight cutoff: 4000−6000). Subsequently, the solvents were removed by using rotary evaporation. The purified polymer appeared as clear oil.

Hydrolytic Degradation of P(MEO_2MA-*co*-OEGMA-*co*-BMDO). In a tube, 1 g of polymer was dissolved in 100 mL of 5% KOH aqueous solution. The mixture was stirred at room temperature for 24 h. After reaction, the mixture was neutralized with 1 N HCl aqueous solution.

Enzymatic Degradation of P(MEO_2MA-*co*-OEGMA-*co*-BMDO). In a test tube, 100 mg of polymer were dissolved in 4 mL of physiological buffer (TRIS or phosphate buffer) and subsequently incubated at 37 °C in the presence of 100 mg of immobilized lipase. After 1 day of incubation, the enzymes were removed from the reaction medium either via filtration (microbeads) or centrifugation (CLEA). All enzymatic degradation tests were compared to control experiments without enzyme (100 mg of polymer in physiological buffer, 24 h, 37 °C). The pH of the buffer solutions was verified to be almost identical in the absence or presence of enzymes.

Cytotoxicity Assays. Metabolic cell damage was determined by means of Luminescence ATP Detection Assay (ATPlite assay form Perkin-Elmer) on the human hepatocellular carcinoma (HepG2) cell line. Polymer solutions (20 mg·mL^{-1}) were prepared in fresh medium containing 10% FCS using ultra sound and diluted afterwards. Cells (2.5×10^4 per well) were seeded in 96 well plates (flat clear bottom white, Corning) and incubated in the presence of serum at 37 °C, 5% CO_2. After 24 h, the medium was removed and polymer solutions were added to cells with a final concentration ranging from 10 to 0.01 mg·mL^{-1}. Subsequently, the plates were incubated at 37 °C, 5% CO_2 for an additional 24 h. Last, medium was replaced with 100 μL PBS and cells were harvested thereafter. Toxicity measurements were done according to the manufacturer's instruction. The data reported in Figures 2 and S1 were measured at 37 °C for polymer concentrations of 5 mg·mL^{-1}. In the studied conditions, the terpolymers P(MEO_2MA-*co*-OEGMA-*co*-BMDO) were not notably hydrolyzed.

Size Exclusion Chromatography, SEC. Molecular weights and molecular weight distributions were determined by SEC performed at 25 °C in tetrahydrofuran (THF) as eluent, using three 5 μ-MZ−SDV columns with pore sizes of 10^3, 10^5, and 10^6 Å (flow rate 1 mL·min^{-1}). The detection was performed with a RI- (Shodex RI-71) and a UV-detector (TSP UV 1000; 260 nm). For calibration, linear polystyrene standards (PSS, Germany) were used.

Cloud Point Measurements. The cloud points of the polymer solutions in water were measured on a Tepper TP1 photometer (Mainz, Germany). Transmittance of polymer solutions in deionized water at 670 nm was monitored as a function of temperature (cell path length = 12 mm; one heating/cooling cycle at rate of 1 °C·min^{-1}).

^{1}H NMR. ^{1}H NMR spectra were recorded in acetone-d_6 on a Bruker DPX-400 operating at 400.1 MHz. Monomer conversions were calculated from ^{1}H NMR spectra. For BMDO, the integration of the CH_2−O protons of the remaining monomer (5.06 ppm) was compared to the overall integration of the region 6.80-7.60 ppm where resonate the aromatic protons of the remaining monomer and of the formed polymer. For MEO_2-MA and OEGMA, an overall monomer conversion was calculated since the double bonds of both monomers have a nearly identical NMR signature. In this case, the conversion was calculated by comparing the integrations of the ethylenic protons of the remaining monomers (5.53 and 6.07 ppm) to the overall integration of the region 3.95−4.35 ppm where resonate two protons of the remaining monomers and two protons of the formed polymers.

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Supporting Information Available: Figure showing additional cytotoxicity measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

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