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Facile Synthesis of Innocuous Comb-Shaped Polymethacrylates with PEG Side Chains by Nitroxide-Mediated Radical Polymerization in Hydroalcoholic Solutions

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ABSTRACT: The nitroxide-mediated copolymerization of poly(ethylene glycol) methyl ether methacrylate (MePEGMA) with a small amount of acrylonitrile using an SG1-based alkoxyamine initiator was shown to be a very simple and efficient technique to synthesize graft copolymers with poly(ethylene glycol) side chains. The copolymerizations were carried out in ethanol/water solutions as environmentally friendly media. Following our observation that the rate increased with the proportion of water, a polymerization temperature as low as 71 °C could be used for a water content of 75%, which conferred great flexibility to the process. The so-formed copolymers were living, with high crossover efficiency toward block copolymers. Importantly, following a cytotoxicity study over three different cell lines that represent important mammalian cell types, these polymers were shown to be noncytotoxic even at very high doses without any other purification step than a simple precipitation. These comb-shaped PEG-based polymers may represent an ideal platform for the synthesis of PEGylating moieties for proteins and nanoparticles intended to be used in the biomedical field.

Introduction

One of the most significant contributions witnessed in polymer science during the past few decades is certainly the development of controlled/living radical polymerization (CLRP) techniques whereby tailor-made (co)polymers and complex macromolecular architectures can be synthesized under rather mild conditions and certain ease.[1−12] Among available polymerization techniques, nitroxide-mediated polymerization (NMP) is historically the first and represents maybe the easiest CLRP technology to apply.[1] NMP is based on a reversible activation−deactivation equilibrium in which the nitroxide reversibly deactivates the growing radical into an alkoxyamine dormant end-functionality. Compared to other CLRP techniques, such as atom transfer radical polymerization (ATRP)[2−4,10] and reversible addition−fragmentation chain transfer (RAFT),[5−7,9] NMP could represent the technique of choice regarding biological applications as it has the advantage of being only governed by a thermal process and does not require a transition metal catalyst or bimolecular exchange with sulfur-based compounds. However, NMP has been for a long time unable to control the polymerization of methacrylic ester monomers, whatever the nitroxide structure.[13−17] With the second generation of nitroxides such as SG1 (*N*-*tert*-butyl-*N*-(1-diethylphosphono-2,2-dimethylpropyl) nitroxide, Scheme 1), this failure is due to unfavorable kinetic and thermodynamic parameters; i.e., the activation−deactivation equilibrium constant, K, is too high. This situation leads to a high concentration of propagating radicals, enhancing the occurrence of irreversible terminations, by both homotermination and β-hydrogen transfer from the propagating radical to the nitroxide.[18−20]

Recently, two distinct approaches have been developed in order to fulfill all the criteria of the controlled/living system while performing NMP of methacrylic ester monomers. The first strategy employs a high dissociation rate constant SG1-based alkoxyamine (2-methyl-2-[*N*-*tert*-butyl-*N*-(1-diethoxyphosphoryl-2,2-dimethylpropyl)aminoxy]propionic acid, BlocBuilder, Scheme 1) and the addition of a very small amount (4.4−8.8 mol %) of styrene as a comonomer.[18] This leads to the preferential formation of more stable macroalkoxyamines/dormant chains with a methacrylate−styrene−SG1 terminal sequence, which are able to dissociate at low temperature (typically < 90 °C). Another consequence is the drastic decrease of the overall concentration of propagating radicals via a drop of the average activation−deactivation equilibrium constant, $\langle K \rangle$.[18,21,22] The second approach uses nitroxides specific to methyl methacrylate (MMA).[23,24] Although the latter strategy permitted the synthesis of controlled PMMA, the copolymerization approach (i) takes advantage of the versatility of the SG1 nitroxide regarding the range of monomers that can be polymerized and (ii) allows well-defined (amphiphilic) block copolymers to be prepared by chain extension.[25,26] Indeed, this rather universal method was successfully applied to MMA,[18,21,25,27] methacrylic acid (MAA),[26−28] and many other methacrylic esters.[29−32]

Poly(ethylene glycol) (PEG) is a key material widely employed in the pharmaceutical area for drug delivery purposes such as polymer−protein/peptide bioconjugates or for the design of "stealth" nanoparticles (this approach is usually termed

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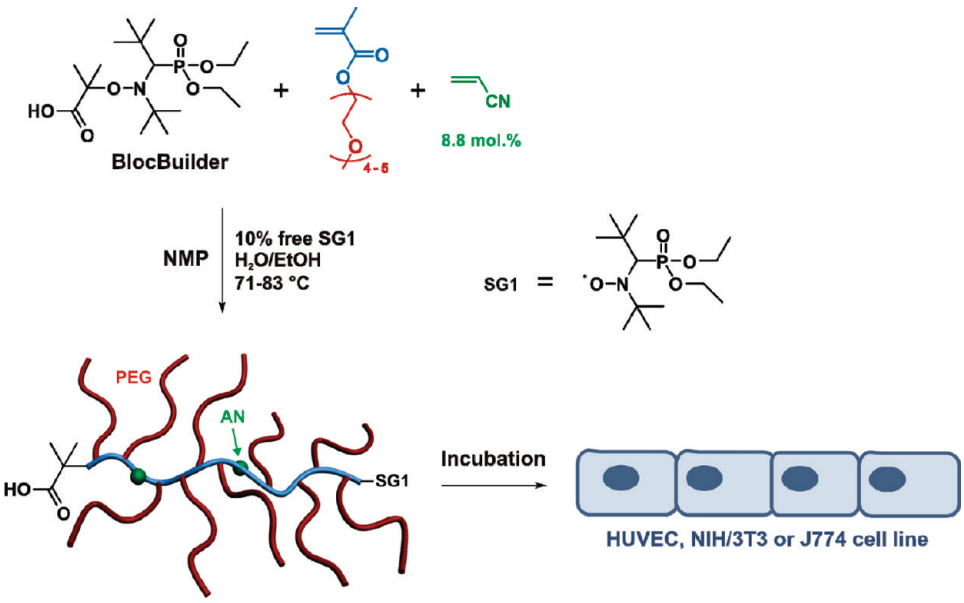
Scheme 1. Synthetic Strategy To Prepare Well-Defined Comb-Shaped Polymethacrylates with Poly(ethylene glycol) Side Chains by Nitroxide-Mediated Polymerization (NMP) and Incubation of the Copolymers with Different Cell Lines for Cytotoxicity Studies



PEGylation).[33−39] Indeed, PEG provides increased bioavailability and plasma half-lives as well as biocompatibility and reduces proteolysis, together with enhanced solubility and stability.[33] Whereas linear PEG have been commonly employed for PEGylation, the rapid growth of CLRP techniques has brought about a significant renewal as new types of PEG-based polymers with unique properties have been synthesized. In this field, the most significant examples are the polymerization of poly(ethylene glycol) methyl ether methacrylate (MePEGMA)[25,29,40−51] or poly(ethylene glycol) methyl ether acrylate (MePEGA)[52,53] which lead to comb-shaped poly(meth)acrylates with PEG side chains. Interestingly with this type of monomer, two degrees of freedom are available as not only the polymer backbone could be nicely controlled (due to the CLRP process) but also the PEG chain length simply by purchasing the appropriate monomer, thus allowing great flexibility regarding macromolecular synthesis. Besides, in the protein PEGylation landscape, it is commonly accepted that the use of branched/comb-shaped PEG polymers in place of linear ones having similar molar masses can, in some cases, provide a number of benefits such as resistance to proteolysis or reduced antibody production which resulted in a lower immunogenicity due to the so-called "umbrella-like" effect.[37,54,55]

Studies dealing with NMP of MePEGMA[29] or MePEGA[53] are quite limited. For instance, the previously mentioned SG1-mediated copolymerization approach with styrene was applied to MePEGMA in ethanol solution at 78.5 °C where it was shown that nicely controlled and living 7−25 kDa comb-shaped polymethacrylates with PEG side chains could be obtained.[29] However, the presence of styrene units within the polymer structure (even though it deals with rather low amounts) could be potentially problematic regarding biological applications as it can create hydrophobic interactions and might be a toxicological issue.

Herein, we propose to take advantage of a very recent study that successfully reported the use of acrylonitrile (AN) as an alternative comonomer for the synthesis of well-defined and living PMMA-rich copolymers under SG1 control.[56] Indeed, contrary to styrene, AN is highly water-soluble. Moreover, a rough extrapolation from poly(alkyl cyanoacrylate) polymers/nanoparticles (which are widely used for drug delivery applications)[57,58] let us conclude that nitrile moieties, when inserted in a polymer structure, are innocuous. In the present study, a comprehensive kinetic investigation of the SG1-mediated solution copolymerization of MePEGMA with a very small amount of AN is performed in order to determine optimal experimental conditions to allow all the criteria of a controlled/living system to be fulfilled (Scheme 1). Polymerizations were conducted in environmentally friendly, hydroalcoholic mixtures where the influence of important parameters such as the initial amount of water over the polymerization rate and the quality of control will be highlighted. In the second part, an *in vitro* cytotoxicity study of P(MePEGMA)-rich copolymers, differing in the nature of the comonomer, either styrene or acrylonitrile, will be presented via cell viability assays and cell morphology observations over three different cell lines. Interestingly, this will help us to determine preliminary polymer structure−cytotoxicity relationships that are of crucial importance regarding forthcoming biological applications. Such a study intends to pave the way to the synthesis of water-soluble polymers to be used as PEGylating moieties for pharmaceutical applications (i.e., PEGylation of either protein therapeutics or nanoparticles). In this context, simplicity of the synthetic and purification procedures, potential for surface chemical modification, innocuousness of the chemical components, and biocompatibility of the final structure are highly desirable.

Experimental Part

1. Materials. Poly(ethylene glycol) methyl ether methacrylate (MePEGMA, M_n = 300 g mol^{-1}), styrene (S, 99%), and acrylonitrile (AN, 99+%) were purchased from Aldrich and used as received. Ethanol absolute (analytical grade) was purchased from Carlo Erba. The 2-methyl-2-[*N*-*tert*-butyl-*N*-(1-diethoxyphosphoryl-2,2-dimethylpropyl)aminoxy]propionic acid alkoxyamine (BlocBuilder MA, 99%) and the *N*-*tert*-butyl-*N*-(1-diethylphosphono-2,2-dimethylpropyl) nitroxide (SG1, 85% purity used for polymerization experiments and 96% purity used for cytotoxicity assays) were kindly supplied by Arkema.

2. Synthesis of P(MePEGMA-*co*-AN)-SG1 Copolymers. A typical solution polymerization procedure (Table 1, experiment 4) was as follows. In a 100 mL three-neck round-bottom flask fitted with a reflux condenser, a magnetic stirrer, a nitrogen inlet, and a thermometer, a mixture of MePEGMA (18.76 g, 0.063 mol), AN (0.324 g, 6.11 × 10^{-3} mol), free SG1 (0.0204 g, 5.97 × 10^{-5} mol), ethanol (22 g), and deionized water (22 g) was

Table 1. Experimental Conditions for the SG1-Mediated Solution Copolymerization of Poly(ethylene glycol) Methyl Ether Methacrylate (MePEGMA) and Acrylonitrile (AN)

					solvent				
expt	monomers[a] (wt %)	MePEGMA[b] (mol L^{-1})	AN (mol L^{-1})	f_{AN0}[c] (mol %)	alcohol	%$_{water}$[d]	temperature (°C)	alkoxyamine[e] (mol L^{-1})	r[f]
1	30	0.99	0.097	8.9	ethanol	0	78.5	8.9×10^{-3}	0.10
2	30	1.00	0.097	8.8	propan-1-ol	0	90	8.9×10^{-3}	0.10
3	30	0.88	0.085	8.8	ethanol	25	83	7.9×10^{-3}	0.10
4	30	0.89	0.087	8.9	ethanol	50	83	7.9×10^{-3}	0.10
5	30	0.93	0.090	8.8	ethanol	75	83	8.3×10^{-3}	0.10
6	30	0.91	0.088	8.8	ethanol	90	83	8.1×10^{-3}	0.10
7	30	0.91	0.089	8.9	ethanol	50	83	8.2×10^{-3}	0.10
8	30	0.96	0.091	8.7	ethanol	75	71	8.5×10^{-3}	0.10
9	30	0.95	0.021	2.2	ethanol	50	83	8.7×10^{-3}	0.10
10	30	0.94	0.045	4.5	ethanol	50	83	8.6×10^{-3}	0.10
11	50	1.59	0.15	8.8	ethanol	50	83	1.5×10^{-2}	0.10
12	15	0.45	0.045	9.1	ethanol	50	83	4.2×10^{-3}	0.10
13	30	0.92	0.090	8.9	ethanol	50	83	1.8×10^{-2}	0.10
14	30	0.90	0.087	8.9	ethanol	50	83	4.0×10^{-3}	0.10
15	30	0.92	0.089	8.8	ethanol	50	83	8.2×10^{-3}	0.25
16	30	0.92	0.089	8.8	ethanol	50	83	8.2×10^{-3}	0.50

[a] Initial overall weight fraction of monomers (τ_0). [b] $M_n = 300$ g mol^{-1}. [c] Initial molar fraction of AN. [d] Weigth percentage of water in the EtOH/water mixture. [e] The BlocBuilder alkoxyamine has been turned into its carboxylate salt form prior introduction except for experiments 3 and 7. [f] Excess of free SG1 = $[SG1]_0/[alkoxyamine]_0$.

deoxygenated by nitrogen bubbling for 20 min at room temperature. The alkoxyamine initiator (0.213 g, 5.59×10^{-4} mol) was dissolved in NaOH 0.4 N (1.95 mL) and added to the reaction medium. Nitrogen bubbling was carried out for 10 min, and the mixture was heated at 83 °C in a preheated oil bath. The time zero of the reaction was triggered when temperature in the reactor reached 75 °C. Samples were periodically withdrawn to follow MePEGMA conversion by ^{1}H NMR spectroscopy. Copolymers were then precipitated twice in cold diethyl ether and dried under high vacuum until constant weight. Conversion (6 h) = 82%, $M_{n,SEC} = 18\,710$ g mol^{-1}, $M_w/M_n = 1.35$ (see Figure 2 for kinetic data).

3. Synthesis of P(MePEGMA-*co*-AN)-*b*-PS Block Copolymer by Chain Extension. Synthesis of the P(MePEGMA-*co*-AN)-*b*-PS block copolymer was as follows. The P(MePEGMA-*co*-AN)-SG1 macroalkoxyamine **17** (1.185 g, 1.13×10^{-4} mol) and styrene (9.0 g, 0.087 mol) were placed in a Schlenk tube and sealed with a rubber septum. The mixture was then deoxygenated by nitrogen bubbling during 30 min, and the Schlenk tube was placed in an oil bath preheated at 120 °C, triggering the beginning of the polymerization. After 8 h, the polymerization was stopped by cooling the reaction medium. Styrene conversion was determined by ^{1}H NMR spectroscopy, and the copolymer was precipitated in hexane and dried under high vacuum until constant weight. $M_{n,SEC} = 54\,900$ g mol^{-1}, $M_w/M_n = 2.3$.

4. Analytical Techniques. ^{1}H NMR spectroscopy was performed in 5 mm diameter tubes in $CDCl_3$ on a Bruker Avance-300 (300 MHz) spectrometer. The chemical shift scale was calibrated on the basis of the solvent peak ($\delta = 7.26$ ppm). ^{13}C NMR spectroscopy was performed in 10 mm diameter tubes in $CDCl_3$ at 25 °C on a Bruker Avance III 500 spectrometer, operating at a frequency of 125.76 MHz. Spectra were recorded applying the following conditions, allowing quantitative analysis: spectral width 248 ppm with 64K data points, flip angle of 30°, relaxation delay of 15 s, digital resolution of 0.47 Hz pt^{-1}, and suppression of the NOE. The chemical shift scale was calibrated on the solvent peak ($\delta = 77.0$ ppm). Size exclusion chromatography (SEC) was performed at 30 °C with two columns from Polymer Laboratories (PL-gel MIXED-D; 300 × 7.5 mm; bead diameter: 5 μm; linear part: $400-4\times 10^5$ g mol^{-1}) and a differential refractive index detector (Spectrasystem RI-150 from Thermo Electron Corp.). The eluent was chloroform ($CHCl_3$) at a flow rate of 1 mL min^{-1}, and toluene was used as a flow-rate marker. The calibration curve was based on poly(methyl methacrylate) (PMMA) standards (peak molar masses, $M_p = 625-625\,500$ g mol^{-1}) from Polymer Laboratories. This technique allowed M_n (the number-average molar mass), M_w (the weight-average molar mass), and M_w/M_n (the polydispersity index, PDI) to be determined.

5. Cell Culture. Embryonic murine fibroblast (NIH/3T3) and human endothelial umbilical vein cells (HUVEC) were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza, Belgium) supplemented with 50 U mL^{-1} penicillin, 50 U mL^{-1} streptomycin, and 10% fetal bovin serum (FBS, Lonza, Belgium). The J774.A1 murine macrophage−monocyte cell line was cultured in RPMI 1640 medium (Lonza, Belgium) supplemented with 50 U mL^{-1} penicillin, 50 U mL^{-1} streptomycin, and 10% heat inactivated FBS. All cell lines were obtained from ATCC and were maintained at 37 °C and 5% CO_2 in a humidified atmosphere.

6. Cell Viability Assay. The *in vitro* cytotoxic activity of the polymers was evaluated on the three cell lines using the 3-[4,5-dimethylthiazol-2-yl]-3,5-diphenyltetrazolium bromide (MTT) test. This assay is based on the cellular reductive capacity to metabolize the MTT to a highly colored formazan product.[59] Cells were seeded in 100 μL of growth medium (HUVEC and J774.A1 8×10^4 cells mL^{-1}, NIH/3T3 4×10^4 cells mL^{-1}) in 96-well microtiter plates (TPP, Switzerland) and preincubated for 24 h. After appropriate dilution, 100 μL of copolymers solution in cell medium was added over the cells and incubated for 48 h (NIH/3T3) or 72 h (HUVEC and J774.A1). Initial cell density and incubation time were chosen to allow cells to remain in exponential growth and to undergo two cell-doubling times during the assay. At the end of the incubation period, 20 μL of a 5 mg mL^{-1} MTT (Sigma-Aldrich, Germany) solution in phosphate buffered saline was added to each well. After 2 h of incubation, the culture medium was removed and replaced by 200 μL of dimethyl sulfoxide (ACS grade, BioBasic Inc., France) in order to dissolve the formazan crystals. The absorbance of the solubilized dye, which correlates with the number of living cells, was measured spectrophotometrically with a microplate reader (LAB Systems Original Multiscan MS, Finland) at 570 nm. The percentage of viable cells in each well was calculated as the absorbance ratio between treated and untreated control cells. A similar protocol was followed with SG1 nitroxide (96%) at 0.1 and 0.3 mg mL^{-1} (SG1 is fully soluble in water at such concentrations).

7. Cell Morphology Observation. For optical microscopy observations, cells were seeded on 9.2 cm^2 tissue culture dishes (TPP, Switzerland). After 24 h, they were incubated with copolymer solutions (1 and 10 mg mL^{-1}) or SG1 solutions (0.1 and 0.3 mg mL^{-1}) in cell growth medium. Cells morphology

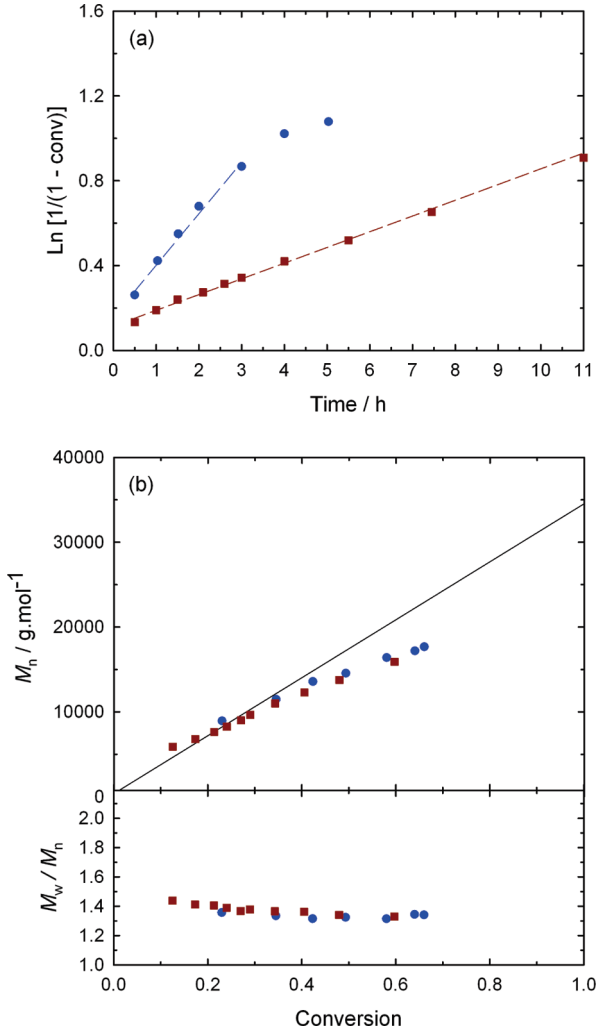


Figure 1. SG1-mediated controlled free-radical solution polymerization of poly(ethylene glycol) methyl ether methacrylate (MePEGMA) with a small amount (f_{AN0} = 0.088) of acrylonitrile (AN) initiated by the BlocBuilder alkoxyamine as a function of solvent and of the polymerization temperature: ■, experiment 1 (ethanol; T = 78.5 °C); ●, experiment 2 (propan-1-ol; T = 90 °C). (a) Ln[1/(1 − conv)] vs time (conv = MePEGMA conversion). (b) Number-average molar mass, M_n, and polydispersity index, M_w/M_n, vs conversion. The full line represents the theoretical M_n and dashed lines represent the best fit of the data.

was observed after 48 h of incubation with a Leitz Diaplan microscope equipped with a Coolsnap ES camera (Roper Scientific). Images were processed using the QED capture software (MediaCybernetics).

Results and Discussion

1. Polymerizations in Alcoholic Solutions. It has been previously shown that replacement of styrene by acrylonitrile for the bulk polymerization of MMA led to strong similarities with regard to kinetics and control.[56] The only major difference was a significantly lower polymerization rate in case of the MMA/AN system, ~5 times lower than in the case of the MMA/S system under similar experimental conditions (i.e., 8.8 mol % of the comonomer and T = 90 °C).

In our experiments, the SG1-mediated copolymerization of MePEGMA with 8.8 mol % AN at 30 wt % solids in ethanol at 78.5 °C (experiment 1, Table 1) proceeded in a controlled fashion but exhibited a rather low polymerization rate (Figure 1), as expected from the above-mentioned kinetic difference. Indeed, even though logarithmic conversion was linear with time and molar masses increased linearly with monomer conversion with low polydispersity indexes (PDIs), only ~60% monomer conversion was reached after 11 h of reaction. For comparison, such a conversion was previously obtained after 8 h with the MePEGMA/S system, all other parameters being kept constant.[29] Note that number-average molar masses, M_n's, increased linearly with monomer conversion, but the slope was lower than the theoretical one due to the conventional calibration used here for the SEC analysis (PMMA standards) that does not give access to absolute M_n's (the theoretical line was nevertheless kept for indication). To circumvent the low polymerization rate, we first considered the use of a higher boiling point alcohol. For this purpose, propan-1-ol (T_{eb} = 97.2 °C at 760 mmHg)[60] was selected and turned out to be suitable for reaching higher polymerization rates together with maintaining a good control. At 90 °C, ~60% monomer conversion was reached after only 3 h with a similar control (experiment 2, Table 1 and Figure 1). However, even though it could represent an efficient solution, increasing the polymerization temperature with the use of longer alkyl chain alcohols might not be the best way from an economical viewpoint. Therefore, we decided to move toward a better alternative by investigating the use of hydroalcoholic mixtures as environmentally friendly media.

2. Polymerizations in Hydroalcoholic Media. *2.1. Variation of the Water Content.* Using water as a solvent is an extremely appealing way for conducting chemical reactions. Indeed, water is inexpensive and environmentally friendly. Additionally for radical polymerization, water is not involved in radical chain transfer reactions due to the high dissociation energy of its O−H bond.[61] Studies reporting NMP in pure water[62−66] or even in homogeneous aqueous mixtures[67−70] are rather limited, and to the best of our knowledge, none of them investigated the polymerization of PEG-based monomers such as MePEGMA or the influence of solvent polarity over the polymerization kinetics.

In this view, solution polymerizations of MePEGMA with the help of 8.8 wt % of AN were performed at 83 °C in ethanol with variable amounts of water (experiments 3−7, Table 1). For high water contents (50−90 wt %), the BlocBuilder alkoxyamine initiator was turned into its carboxylate salt form prior to the introduction in the reaction medium in order to ensure its complete solubilization (see Table 1). From the kinetic point of view, increasing the initial amount of water in the solvent mixture from 25 to 75 wt %, other parameters being kept constant, resulted in a dramatic increase in the polymerization rate as attested by the slopes of the logarithmic conversion versus time plots in Figure 2a. Indeed, experiments 3, 4, and 5 exhibited a slope (which correspond to $\langle k_p \rangle$[P•], with $\langle k_p \rangle$ the average rate constant of propagation in copolymerization and [P•] the overall concentration of propagating radicals) of 3.83 × 10^{-5}, 8.04 × 10^{-5}, and 1.32 × 10^{-4} s^{-1}, respectively. This feature was assigned to the higher polarity of the solvent mixture upon increasing the initial water content, in good agreement with earlier results based on model alkoxyamines.[71,72] In these studies, it was reported that the rate constant of the C−O bond cleavage (k_d) increased with organic solvent polarity (such as hexane, acetonitrile, DMF, etc.). Furthermore, it has been shown that nitroxides interact with polar protic solvents via intermolecular hydrogen bonding, hence leading to lower rate constants of recombination (k_c).[73] These two trends led to a higher equilibrium constant ($K = k_d/k_c$) which, in turn, induced a higher polymerization rate. To date, there is only one example that highlighted the influence of the solvent polarity on the polymerization rate for NMP.

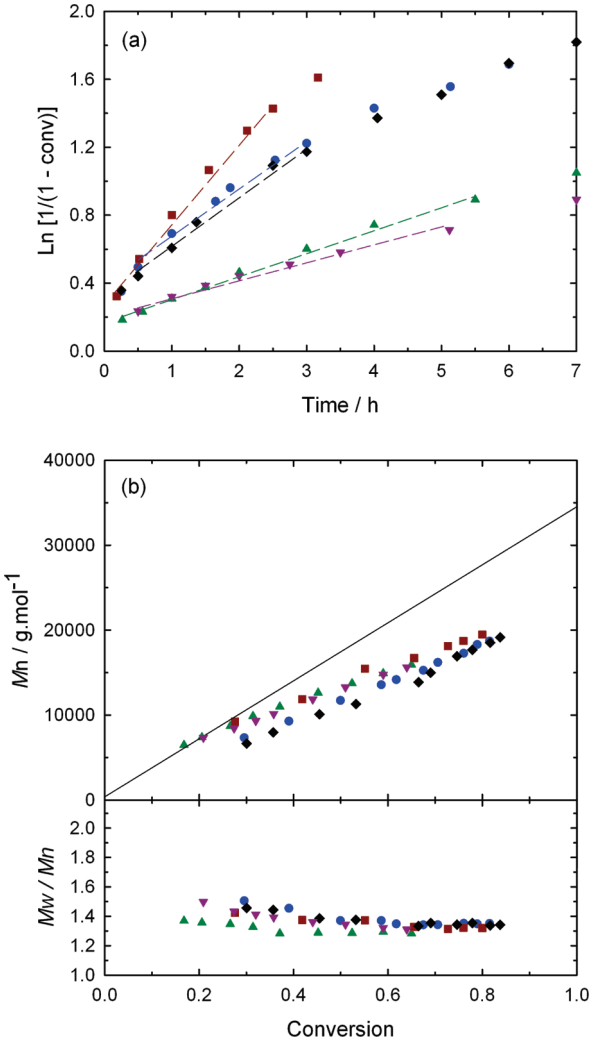


Figure 2. SG1-mediated controlled free-radical solution polymerization of poly(ethylene glycol) methyl ether methacrylate (MePEGMA) with a small amount (f_{AN0} = 0.088) of acrylonitrile (AN) initiated by the BlocBuilder alkoxyamine as a function of the initial amount of water ($\%_{water}$) in the ethanol/water mixture and of the polymerization temperature: ▲, experiment 3 ($\%_{water}$=25; T=83 °C); ●, experiment 4 ($\%_{water}$ = 50; T = 83 °C); ■, experiment 5 ($\%_{water}$ = 75; T = 83 °C); ♦, experiment 7 (identical as experiment 4 but initiated by the acidic form of the BlocBuilder alkoxyamine); ▼, experiment 8 ($\%_{water}$ = 75; T = 71 °C). (a) Ln[1/(1 − conv)] vs time (conv = MePEGMA conversion). (b) Number-average molar mass, M_n, and polydispersity index, M_w/M_n, vs conversion. The full line represents the theoretical M_n and dashed lines represent the best fit of the data.

This was reported by Chiu and co-workers, who pointed out an accelerating effect of DMF during the bulk polymerization of *tert*-butyl acrylate initiated by 4-oxo-TEMPO-capped polystyrene macroinitiators.[74] Another explanation, specific to MePEGMA, could arise from the formation of micellar organizations with the methacrylate moieties confined in hydrophobic domains or clusters, which yielded higher k_p's and highly limited terminations between compartmentalized propagating radicals.[75−77] This particular behavior of PEG-based macromonomer is also explained by the work of Beuermann and co-workers, who reported that methacrylates carrying PEG units follow the trends observed for k_p in the methacrylate family. However, they showed significant deviations from the termination kinetics of alkyl methacrylates and especially a strong reduction of chain-length-averaged termination rate constant $\langle k_t \rangle$ with conversion.[78]

Interestingly, in our case the high monomer conversion (~80%) was reached after only 3 h of reaction when the hydroalcoholic solution contained 75 wt % of water (experiment 5, Figure 2a). Concerning the quality of control, linear evolution of molar masses with monomer conversion was obtained together with rather low PDIs ($M_w/M_n \sim 1.3$) in all cases (Figure 2b). It thus demonstrated the accurate tuning of the polymerization rate together with the lack of any detrimental effect of the addition of water over the control process. However, when the water content was increased up to 90 wt % (experiment 6, Table 1), the reaction medium became cloudy after ~20 min of polymerization and yielded a precipitate after 1 h. Nevertheless, the copolymerization exhibited a linear logarithmic conversion with time, with a slope ($\langle k_p \rangle [P^\bullet] = 1.50 \times 10^{-4}$ s^{-1}) slightly higher compared to experiment 5, and copolymers obtained were of well-defined structure (54% monomer conversion, M_n = 15 900 g mol^{-1}, M_w/M_n = 1.37). This is in total agreement with the well-known lower critical solution temperature (LCST) of PEG,[79,80] which has been observed at around 60 °C for P(MePEGMA) polymers.[81,82] Interestingly, a duplicate polymerization of experiment 4 was also performed by using the BlocBuilder alkoxyamine under its acidic form (experiment 7, Table 1). This had no effect either on the polymerization kinetics or on the control as both experiments were almost perfectly overlaid (Figure 2).

Then, we decided to take advantage of the very high polymerization rate observed for experiment 5 by drastically decreasing the polymerization temperature down to 71 °C while still having 75 wt % of water in the hydroalcoholic mixture (experiment 8, Table 1). Interestingly, a satisfying polymerization rate was obtained, rather similar to its counterpart carried out at 83 °C with 25 wt % of water, while maintaining a good control (Figure 2). Therefore, adjusting the water content in the solvent mixture (i.e., varying the global polarity of the reaction medium) allowed very low polymerization temperature to be reached and great flexibility regarding the polymerization kinetics. It is worth mentioning that a successful NMP (i.e., good control and acceptable polymerization rate), whatever the chosen solvent/initiator/nitroxide/monomer(s) system, has never been performed at such a low temperature. For instance, the bulk copolymerization of MMA and styrene (f_{S0} = 8.8 mol %) at 78 °C gave a monomer conversion of 33% after 5 h,[21] whereas it reached ~50% with our system at 71 °C.

Importantly, styrene cannot be used as a comonomer at such low temperature and with such high amount of water as its copolymerization with MePEGMA (f_{S0} = 8.8 mol %) led to bimodal molar mass distribution (data not shown), likely due to its poor solubility in the solvent mixture compared to acrylonitrile (at 50 °C, the water solubility is 4.3×10^{-3} mol L^{-1} for styrene whereas it is 1.58 mol L^{-1} for acrylonitrile).[83]

2.2. Influence of the Initial Amount of Acrylonitrile and Solid Content. The proportion of water was then maintained at 50 wt %, and the initial molar fraction of AN (f_{AN0}) ranged from 2.2 to 8.9 mol % (experiments 4, 9, and 10, Table 1). As anticipated, the lower the f_{AN0}, the higher the polymerization rate and the final PDI, as shown in Figure 3. This is in total agreement with bulk copolymerization results involving MMA/S[18] and MMA/AN[56] systems. However, unlike those previous studies, reducing the initial molar fraction of AN in the monomer feed as low as 2.2 mol % did not significantly alter the quality of control as the final PDI at ~85% conversion only increased from 1.35 (f_{AN0} = 8.9 mol %) to 1.47 (f_{AN0} = 2.2 mol %). This highlights an important difference with previous approaches for which PDIs usually reached 1.6−1.7 at low monomer conversion

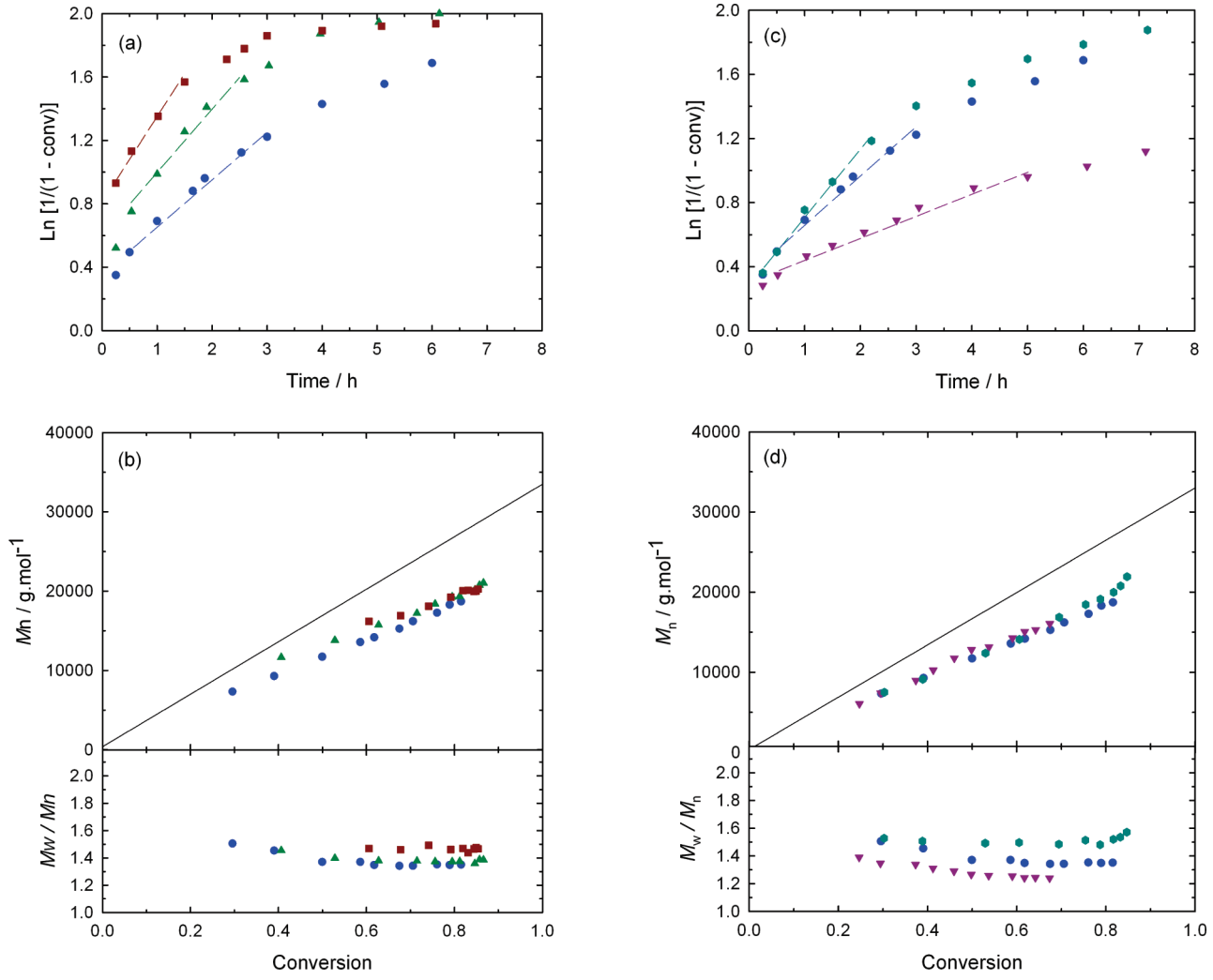


Figure 3. SG1-mediated controlled free-radical solution polymerization of poly(ethylene glycol) methyl ether methacrylate (MePEGMA) at 83 °C in ethanol/water (50/50; v/v) initiated by the BlocBuilder alkoxyamine as a function of the initial molar fraction of acrylonitrile (AN), f_{AN0} (a, b), and the solid content, τ_0 (c, d): ●, experiment 4 (f_{AN0} = 8.9 mol %; τ_0 = 30 wt %); ■, experiment 9 (f_{AN0} = 2.2 mol %; τ_0 = 30 wt %); ▲, experiment 10 (f_{AN0} = 4.5 mol %; τ_0 = 30 wt %); ⬢, experiment 11 (f_{AN0} = 8.8 mol %; τ_0 = 50 wt %); ▼, experiment 12 (f_{AN0} = 9.1 mol %; τ_0 = 15 wt %). (a, c) Ln[1/(1 − conv)] vs time (conv = MePEGMA conversion); (b, d) number-average molar mass, M_n, and polydispersity index, M_w/M_n, vs conversion. The full line represents the theoretical M_n's and dashed lines represent the best fit of the data.

(40−50%) with the same initial amount of comonomer. In other words, the broadening of the molar mass distributions when lowering f_{AN0} was less pronounced with the MePEGMA/AN copolymerization procedure and allowed significantly higher monomer conversions to be reached while still maintaining rather low polydispersity indexes.

Increasing the solids content from 15 to 50 wt % was undertaken (experiments 4, 11, and 12, Table 1) and led to a higher polymerization rate and a broadening of the molar mass distribution (Figure 3), similarly to SG1-mediated copolymerization of MePEGMA and S in ethanol solution.[29] Indeed, even though linear evolutions of molar masses with monomer conversion were shown in all cases, PDI reached 1.6 for 50 wt % solids whereas it was 1.25 for 15 wt %. The impact of increasing concentration of both monomer and alkoxyamine on the polymerization rate could be explained by the effect of the reaction medium (polarity and/or viscosity),[29,84−86] whereas its detrimental influence on the control is in good agreement with the equation describing the persistent radical effect,[87] even though the occurrence of radical chain transfer to PEG cannot be ruled out.

2.3. Influence of the Initial Concentration of Alkoxyamine and Excess of Free SG1. In order to target different molar masses, the initial alkoxyamine concentration was varied to cover a broad range of M_n's (experiments 4, 13, and 14, Table 1). Whatever the targeted molar mass, the evolutions of logarithmic conversion with time exhibited linear parts accounting for a constant concentration of propagating radicals (Figure 4). As already observed,[18,22,56] despite identical $[SG1]_0/[alkoxyamine]_0$ initial ratios which should result in similar polymerization rates (this holds only when the amount of free nitroxide is higher than the amount of released nitroxide due to termination reactions),[88] a significant difference of ln[1/(1 − conv)] vs time plot was however noticed upon decreasing $[alkoxyamine]_0$. The reason for such a trend is due to early irreversible termination reactions, which occurred to a higher extent when $[alkoxyamine]_0$ was decreased. This conducted to a slowdown of the polymerization rate due to the release of extra free SG1. This also explained the sharper decrease of the polydispersity indexes for the smallest $[alkoxyamine]_0$.[88,89] Nevertheless, molar masses increased linearly with monomer conversion while achieving different final molar masses (in good agreement with the $[alkoxyamine]_0$ value) and PDIs as low as ∼1.3.

The initial excess of free SG1 (usually defined as $r = [SG1]_0/[alkoxyamine]_0$) has been often used as a rate moderator,

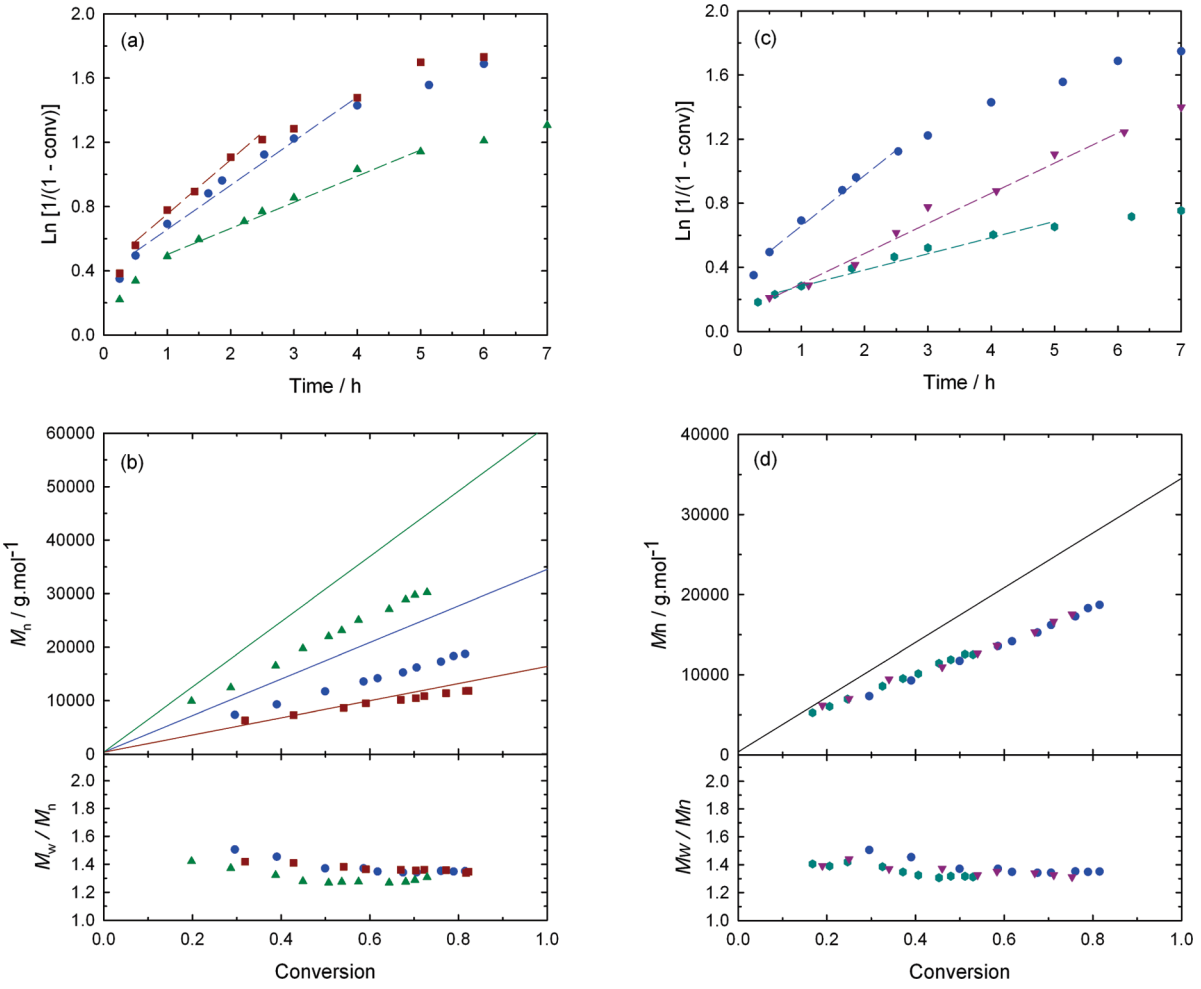


Figure 4. SG1-mediated controlled free-radical solution polymerization of poly(ethylene glycol) methyl ether methacrylate (MePEGMA) at 83 °C in ethanol/water (50:50; v/v) initiated by the BlocBuilder alkoxyamine as a function of the initial alkoxyamine concentration (a, b) and the excess of free SG1, r (c, d): ●, experiment 4 ($[\text{alkoxyamine}]_0 = 7.9 \times 10^{-3}$ mol L^{-1}, $r = 0.10$); ■, experiment 13 ($[\text{alkoxyamine}]_0 = 1.8 \times 10^{-2}$ mol L^{-1}, $r = 0.10$); ▲, experiment 14 ($[\text{alkoxyamine}]_0 = 4.0 \times 10^{-3}$ mol L^{-1}, $r = 0.10$); ▼, experiment 15 ($f_{\text{AN0}} = 8.9$ mol %; $r = 0.25$); ⬢, experiment 16 ($f_{\text{AN0}} = 8.9$ mol %; $r = 0.50$). (a, c) Ln[1/(1 − conv)] vs time (conv = MePEGMA conversion); (b, d) number-average molar mass, M_n, and polydispersity index, M_w/M_n, vs conversion. The full lines represent the theoretical M_n's and dashed lines represent the best fit of data. $r = [\text{SG1}]_0/[\text{alkoxyamine}]_0$.

especially for the polymerization of acrylates.[90,91] Here, r was increased from 0.1 to 0.5 in order to strongly influence the polymerization kinetics (experiments 4, 15, and 16, Table 1). Polymerization behaved as expected: the higher r, the lower the polymerization rate (Figure 4). The evolutions of the molar masses with monomer conversion were linear and perfectly overlaid. A slight improvement of the molar mass distribution was even detected upon increasing r.

Equation 1 was obtained by adapting eq 6 in ref 91 to a copolymerization system, where $\langle k_p \rangle$ is the average propagation rate constant, $[\text{P}^\bullet]$ the overall concentration of propagating radicals, $\langle K \rangle$ the average activation−deactivation equilibrium constant, $[\text{alkoxyamine}]_0$ the initial concentration of alkoxyamine, and $[\text{SG1}]_x$ the concentration of released SG1 at a given monomer conversion x.

$$\frac{1}{\langle k_p \rangle [\text{P}^\bullet]} = \frac{r}{\langle k_p \rangle \langle K \rangle} + \frac{[\text{SG1}]_x}{\langle k_p \rangle \langle K \rangle [\text{alkoxyamine}]_0} \qquad (1)$$

As illustrated in Figure 5, the evolution of $(\langle k_p \rangle [\text{P}^\bullet])^{-1}$ vs r was linear which allowed important kinetic parameters to be extracted such as $\langle k_p \rangle \langle K \rangle$ and $[\text{SG1}]_x/[\text{alkoxyamine}]_0$. The treatment of this equation is actually oversimplified as $[\text{SG1}]_x$ varies with conversion and would be different for

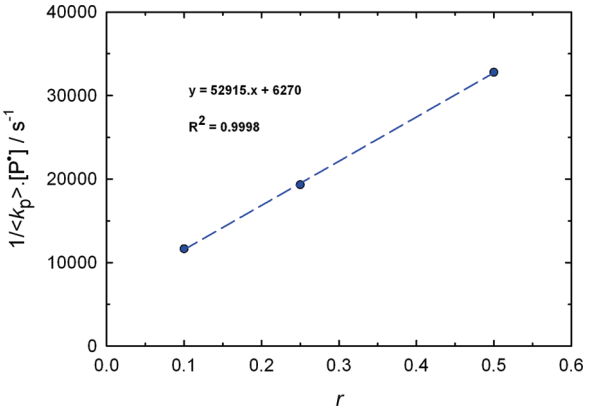


Figure 5. Effect of the initial r value ($r = [\text{SG1}]_0/[\text{alkoxyamine}]_0$) on the rate of polymerization at 83 °C for the SG1-mediated solution copolymerization of poly(ethylene glycol) methyl ether methacrylate (MePEGMA) and acrylonitrile (AN) in ethanol/water (50:50; v/v), initiated by the BlocBuilder alkoxyamine.

different values of r. Nevertheless, by extrapolating to $r = 0$ in a linear fashion, it may give an estimation of the maximum amount of free SG1 released (i.e., the maximum proportion

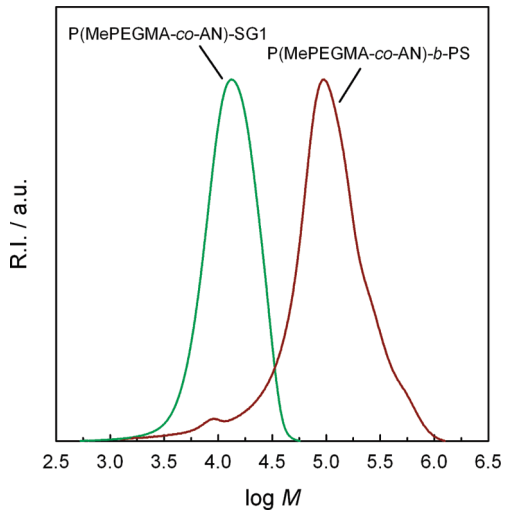


Figure 6. Size exclusion chromatograms of poly[poly(ethylene glycol) methyl ether methacrylate-*co*-acrylonitrile]-*b*-polystyrene (P(MePEGMA-*co*-AN)-*b*-PS) diblock copolymer resulting from chain extensions of the poly[poly(ethylene glycol) methyl ether methacrylate-*co*-acrylonitrile]-SG1 (P(MePEGMA-*co*-AN)-SG1) macroalkoxyamine **17**.

of dead chains) in the polymerization system upon self-termination of the propagating radicals by the persistent radical effect (PRE). In our case, the intercept is 6270 s whereas the slope, $(\langle k_p \rangle \langle K \rangle)^{-1}$, is 52 915 s. From these values, one could calculate $\langle k_p \rangle \langle K \rangle = 1.89 \times 10^{-5}$ s^{-1}, which is in the typical range for achieving a good control,[56] and $[SG1]_x/[alkoxyamine]_0 = 0.12$. The latter result highlights that the proportion of dead chains formed by irreversible homoterminations remained rather low, taking into account the very high proportion of methacrylic ester (> 91 mol %) in the monomer feed. Indeed, even though $[SG1]_x$ are usually in the 0.03−0.05 range for acrylates,[91] our value was comparable to those observed for homopolymerization of styrene under SG1 control.[92]

3. Livingness. Another important criterion for controlled/living radical polymerization is the high living chain fraction that should remain upon polymerization. In this view, a P(MePEGMA-*co*-AN)-SG1 macroalkoxyamine was synthesized (experiment 17) following experimental conditions of experiment 4. However, to ensure a high living chain fraction, the reaction was stopped at low monomer conversion (after purification: 38% monomer conversion; $M_n = 10\,470$ g mol^{-1}, $M_w/M_n = 1.37$). Chain extension was then performed from **17** with styrene at 120 °C for 8 h in order to allow a strong shift of the SEC trace toward a higher molar mass (Figure 6). As expected, a nearly complete shift of the SEC peak was noticed and only a very small peak remained accounting for a small fraction of dead chains, similarly to previous chain extension of SG1-terminated P(MePEGMA-*co*-S)-SG1 copolymer.[25] Interestingly, this was also in good agreement with the low concentration of released SG1 as determined earlier. SEC of the resulting P(MePEGMA-*co*-S)-*b*-PS block copolymer gave $M_n = 54\,900$ g mol^{-1} with a high polydispersity index of 2.3 due to the experimental conditions of the chain extension which were not devoted to a well-controlled growth of the second block.[25]

4. Cytotoxicity Studies. *4.1. Effect of Copolymers on Cell Viability*. Those comb-shaped polymethacrylates with PEG side chains may be useful in the biomedical field by allowing either (i) to perform PEGylation of proteins (providing a reactive function is present within the polymer structure, such as the carboxylic acid group of the BlocBuilder initiator, which can be easily turned into an activated ester)[93] or (ii) to prepare "stealth" (i.e., PEGylated) nanoparticles for drug delivery purposes. Even though a great deal of effort is being devoted to develop ATRP-based processes with lower amounts of catalyst[10] or efficient RAFT end-group removal pathways[94] due to potential toxicological issues, NMP may still represent the safest CLRP technique regarding biomedical and environmentally friendly applications. In this view, prior to any biopharmaceutical use and further clinical application of this PEG-based materials, it is important to assess their safety.[95,96] Copolymers have been tested on different kinds of cells to investigate any cytotoxic effect on the basis of cell viability assays and cell morphology observations. Three cell lines have been used: mouse fibroblast cells (NIH/3T3), human umbilical vein endothelial cells (HUVEC), and murine macrophage cells (J774.A1) which represent important mammalian cell types. The HUVEC cell line has been selected due to its high sensitivity and rapid response to external stimuli. Besides, it is considered to be a good *in vitro* model to evaluate cytotoxicity of different materials.[97,98] The endothelial cells, which line the entire circulatory system within the body, are the first to come into contact with therapeutic systems after intravenous administration. Consequently, their sensitivity to synthetic materials needs to be investigated.[99,100]

Thus, to evaluate *in vitro* cytotoxicity, concentrations of copolymers ranged from 0.1 to 10 mg mL^{-1} in order to ensure their safe *in vivo* administration regarding therapeutic applications. It is important to mention that 10 mg mL^{-1} is an extremely high concentration that does not reflect usual amounts of therapeutics that would be administrated during clinical trials or biomedical assays. The purpose of such a very high dose was only to overexpress any cytotoxic effect that could arise from the nature of the monomer(s) (and therefore the corresponding copolymer) and/or from the presence of the SG1 terminal group. For such a study, two comb-shaped polymethacrylates with PEG side chains copolymers have been selected, only differing by the nature of the comonomer (see Table 2): either styrene (experiment 18) or acrylonitrile (experiment 19). In both cases, protocol of experiment 4 was followed and a final molar mass of about 10 kg mol^{-1} was targeted.

Investigation of copolymers' cytoxicity was performed by MTT assay which is a relevant method for synthetic (co)polymers.[101] Average cell viabilities following incubation with the copolymers are reported in Figure 7. Exposition of the three cell lines to both copolymer **18** and **19** at concentration of 0.1 and 1 mg mL^{-1} did not affect the cell viability compared to untreated cells. This result is of high importance as it shows the safeness of those copolymers under already high concentrations. Interestingly, only the highest concentration of 10 mg mL^{-1} allowed both the sensitivity of the three cell lines regarding the copolymers and the influence of the copolymer structure over cell viability to be discriminated. After 72 h of incubation, the HUVEC cells showed a moderate reduction of viability, which remained however still as high as 76 ± 2% without significant difference between styrene- and acrylonitrile-containing copolymers (Figure 7b). In our case, none of the samples, even at the highest concentration, showed any significant cytotoxic effect on HUVEC cells which may be considered as an important result.

On the contrary, at the same concentration (i.e., 10 mg mL^{-1}), copolymers led to marked difference in cell viability with NIH/3T3 and J774.A1 cell lines. It is worth mentioning that the higher cytotoxicity on J774.A1 and NIH/3T3 cell

Table 2. Macromolecular Characteristics of the MePEGMA-Based Copolymers Synthesized by SG1-Mediated Solution Copolymerization for Cytotoxicity Studies

expt	copolymer	polymerization time (min)	monomer conversion (%)	M_n[a] (g mol^{-1})	PDI[a]	av composition of the copolymer (comonomer) F_M
18	P(MePEGMA-*co*-S)-SG1	20	34	10 150	1.56	0.128 (S)[b]
19	P(MePEGMA-*co*-AN)-SG1	30	38	13 630	1.41	0.069 (AN)[c]

[a] Determined by SEC in chloroform with PMMA standards. [b] Determined by ^{1}H NMR spectroscopy. [c] Determined by ^{13}C NMR spectroscopy.

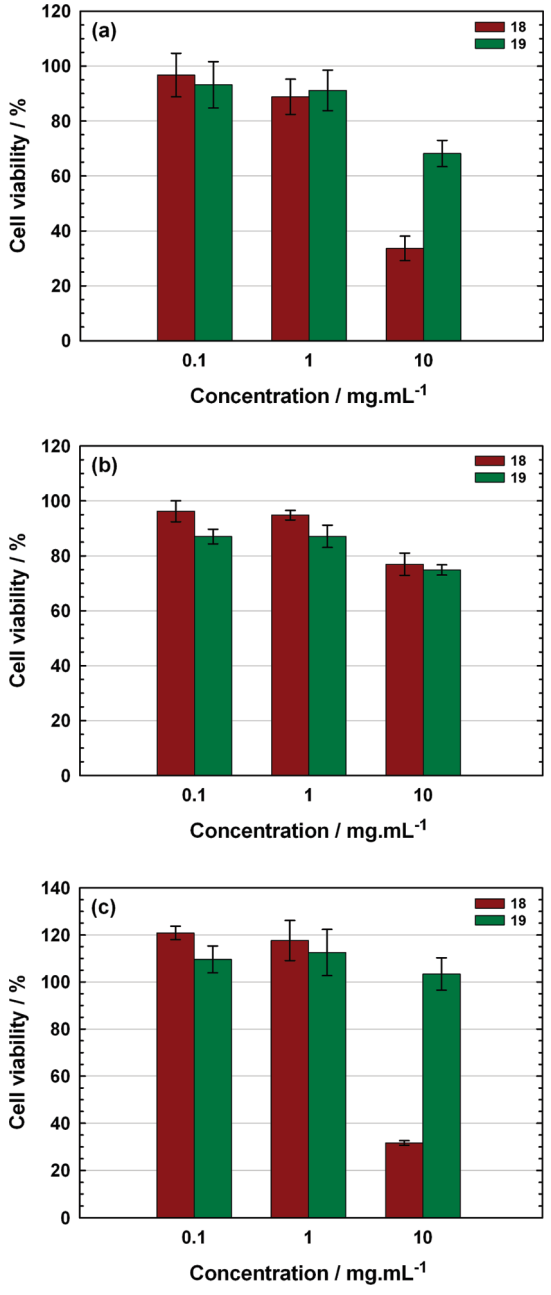


Figure 7. Cell viability (MTT assay) after incubation of NIH/3T3 cells (a), HUVEC cells (b), and J774.A1 cells (c) with poly[poly(ethylene glycol) methyl ether methacrylate-*co*-styrene]-SG1 (P(MePEGMA-*co*-S)-SG1, **18**) and poly[poly(ethylene glycol) methyl ether methacrylate-*co*-acrylonitrile]-SG1 (P(MePEGMA-*co*-AN)-SG1, **19**) copolymer as a function of the copolymer concentration. Each experiment was repeated eight times from four independent incubation preparations. Results were expressed as percentages of absorption of treated cells (±SD) in comparison with the values obtained from untreated control cells.

lines compared to HUVEC cells was likely due to their relatively higher ability, especially for macrophagic cells, to capture extracellular material.[102−104] Indeed, whereas copolymer **19** yielded completely viable J774.A1 cells and only slight cytoxicity on NIH/3T3 cells, incubation of copolymer **18** led to significant cell death as cell viability drastically dropped down to 34 ± 4 and 32 ± 1%, respectively (Figure 7a,c). Even though this severe cytotoxicity clearly came from the use of styrene compared to acrylonitrile, the origin of the toxic effect is far more complex to assign. Cytotoxicity may be due to (i) aromatic rings of styrene units within the copolymer structure, leading to hydrophobic interactions with various cell components, and/or (ii) a very slow release of macroradicals and free SG1 as a consequence of the macroalkoxyamine homolytic cleavage that could occur over a long period of time, even at such low temperature (i.e., 37 °C for 48 or 72 h). Regarding detrimental effect of radicals on cell viability, it is indeed well-known that radical species can interact with oxygen to form oxygen reactive species such as peroxides that induce cell death due to oxidative stress.[105,106] It is well-established for model alkoxyamines such as phenylethyl-SG1 and propionitrile-SG1 (that would mimic the S-SG1 and AN-SG1 terminal sequences, respectively) that the former is much less stable in solution than the latter.[107,108] Even in copolymers for which penultimate methacrylate unit may enhance the homolytic cleavage, the relative effect would be the same as supported by the much faster polymerization reaction for the methacrylate/styrene systems as compared with the methacrylate/acrylonitrile ones (see ref 56 and this work). However, even though a release of carbon-centered radicals could explain the strong differences of cell viability, the effect of hydrophobic interactions of styrene units cannot be ruled out. Nevertheless, the change from styrene to acrylonitrile as a comonomer almost entirely suppressed cytotoxicity and allowed extremely high copolymer concentrations to be safely reached. As a consequence, with selection of appropriate monomers, this technique can readily lead to original and safe materials for application in nanomedicine.

4.2. Effect of Copolymers on Cell Morphology. Any modification of cell morphology was then microscopically monitored (see Figure 8) after a 48 h exposure to the copolymers at the two highest concentrations (1 and 10 mg mL^{-1}). In very good agreement with cell viability assays, incubation of HUVEC cells with both copolymers **18** and **19** did not alter the cell cobblestone morphology (Figure 8f−j). They fully spread and were morphologically indistinguishable from untreated cells. Similar observations were obtained with J774.A1 and NIH/3T3 cell lines upon incubation with both copolymers at a concentration of 1 mg mL^{-1}, where cells proliferation proceeded normally. J774.A1 cells have uniform smooth appearance, and no difference was detectable regarding cell size and density compared to untreated cells (Figure 8l,m), whereas NIH/3T3 cells grew and spread perfectly forming sheets that firmly adhered to the culture dish, leading to an entirely coverage of the surface (Figure 8b,c).

In contrast, incubation with the highest concentration (10 mg mL^{-1}) led to appreciable cell morphological transformations. Figure 8n shows J774.A1 cells treated with copolymer

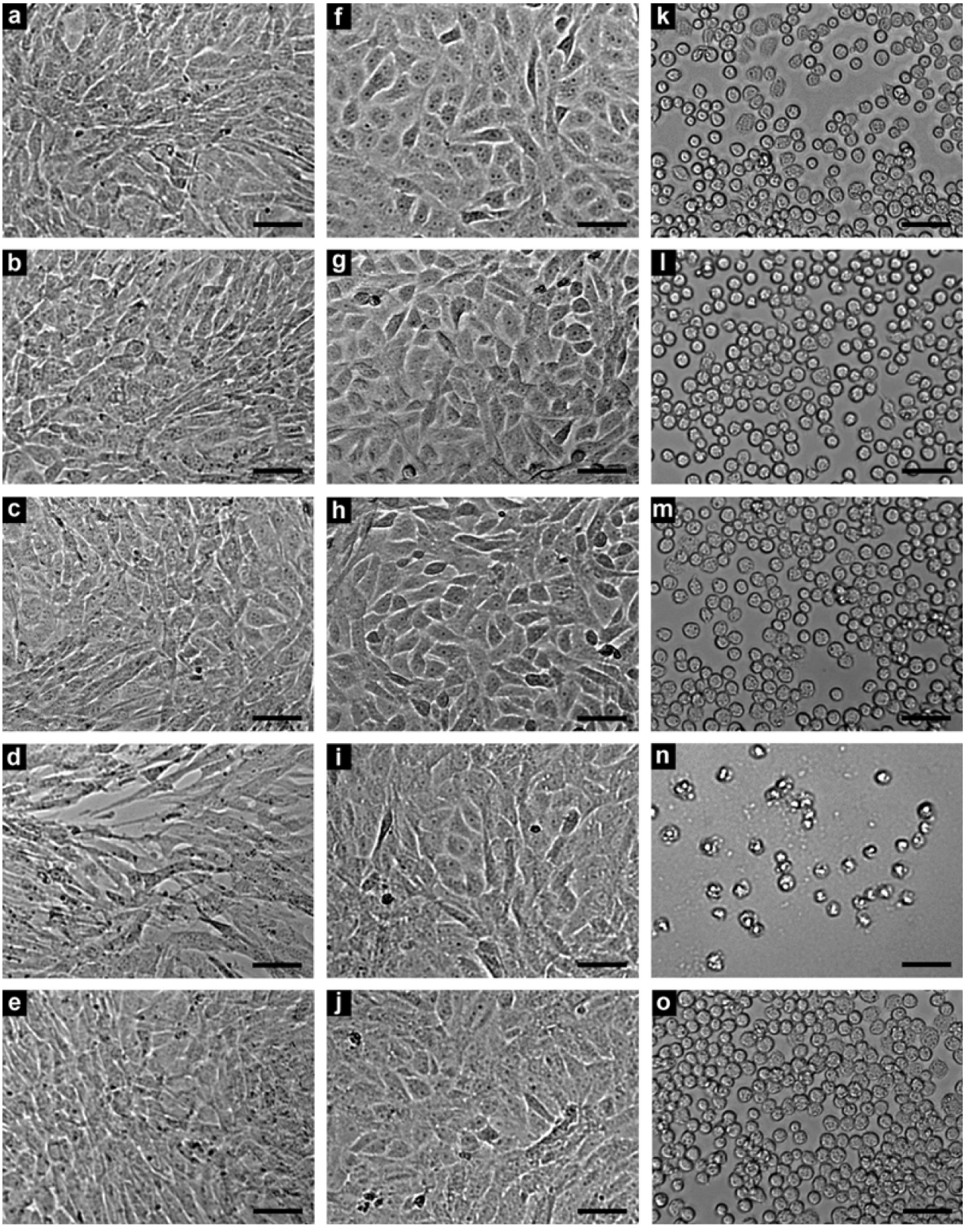


Figure 8. Morphology of NIH/3T3 cells (first column, a−e), HUVEC cells (second column, f−j), and J774.A1 cells (third column, k−o) monitored by optical microscopy before (line 1) and after treatment for 48 h with copolymer poly[poly(ethylene glycol) methyl ether methacrylate-*co*-styrene]-SG1 (P(MePEGMA-*co*-S)-SG1, **18**), at 1 mg mL^{-1} (line 2), poly[poly(ethylene glycol) methyl ether methacrylate-*co*-acrylonitrile]-SG1 (P(MePEGMA-*co*-AN)-SG1, **19**) at 1 mg mL^{-1} (line 3), P(MePEGMA-*co*-S)-SG1 (**18**) at 10 mg mL^{-1} (line 4), and P(MePEGMA-*co*-AN)-SG1 (**19**) at 10 mg mL^{-1} (line 5). Scale bar = 50 μm.

18, confirming the severe cytotoxicity observed in cell viability assay where styrene-containing copolymer caused marked cell death with a reduction of cell viability of about 70%. By microscopy, only sparse cells exhibiting shrinking membranes were still present. Dead cells did lose their adhering capacity, detached from the culture plate and were

readily rinsed off. Similarly, morphological modifications with NIH/3T3 cells only appeared after treatment with styrene-containing copolymer **18** (Figure 8d). A reduction of cell density was observed, and the cellular bodies became thinner compared to physiological conditions. Thus, with NIH/3T3 cells, morphological examination allowed also correlation with the reduction of cell viability induced by **18** (see Figure 7a). These observations do not yet allow to discriminate between necrosis, apoptosis, or other cell death mechanisms,[109] induced by **18** which deserves further investigation. In contrast, with copolymer **19**, no morphological change was observed at 10 mg mL^{-1} (Figure 8o), which clearly evidenced the safe profile of this material.

4.3. Cytotoxicty of the SG1 Nitroxide. Following these results, it appeared crucial here to investigate any cytotoxicity coming from the SG1 nitroxide in order to partially tackle the origin of the reduced cell viability upon exposure to styrene-containing copolymer. Therefore, a similar cytotoxicity study was performed with NIH/3T3 and J774.A1 cells (i.e., cell lines which led to significant reduction of cell viability upon incubation with P(MePEGMA-*co*-S)-SG1 at 10 mg mL^{-1}). Cells were incubated with free SG1 solutions at 0.1 and 0.3 mg mL^{-1}, that would mimic ~30% and ~100% of released SG1 from copolymer **18** at 10 mg mL^{-1}, respectively. MTT assay clearly indicated that free SG1 did not affect cell viability for both concentrations and on both cell lines (Figure 9). These results were fully corroborated by direct microscopic observation, which revealed no changes in morphological appearance and cell density (Figure 10). As a consequence, the lack of any cytotoxic effect coming from free SG1 established for the first time the innocuousness of NMP using SG1 as a control agent.

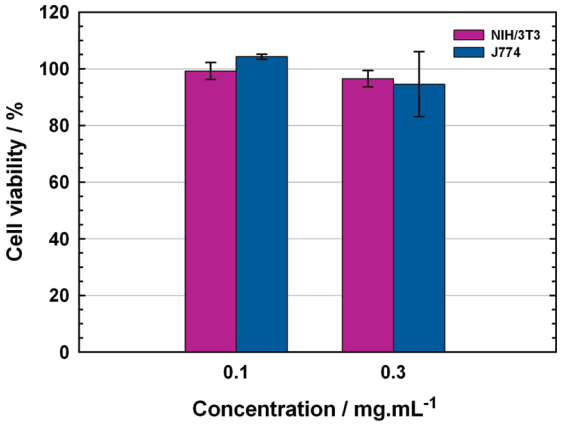


Figure 9. Cell viability (MTT assay) after incubation of NIH/3T3 cells and J774.A1 cells with free SG1 as a function of the SG1 concentration. Each experiment was repeated eight times from four independent incubation preparations. Results were expressed as percentages of absorption of treated cells (±SD) in comparison with the values obtained from untreated control cells.

Conclusion

The nitroxide-mediated polymerization based on the nitroxide SG1 was shown to be a simple and efficient technique to synthesize graft copolymers with poly(ethylene glycol) side chains. Because of the use of macromonomers with a methacrylate reactive group, which are readily available and do not undergo side reactions during radical polymerization, the NMP method requires the use of a low amount of an appropriate comonomer. It was shown here that acrylonitrile is efficient, and the method could be performed at very low temperature for NMP in water-rich hydroalcoholic solutions. The so-formed copolymers were living; they exhibited a carboxylic acid group at the alpha chain end for further coupling of biologically active molecules. Besides, they were shown to be noncytotoxic without any other purification step than a simple precipitation. To date, this represents the first cytotoxicity study of polymers synthesized by NMP, demonstrating the innocuousness of the method. Furthermore, because of the alkoxyamine reactive end group, such water-soluble copolymers can be chain-extended in water to yield block copolymer nanoparticles.[25] As a consequence, they show interesting prospects as a platform with regards to the synthesis of PEGylating moieties for proteins and nanoparticles intended to be used for biomedical applications. Such a study is currently under progress and will be shortly the topic of a forthcoming article.

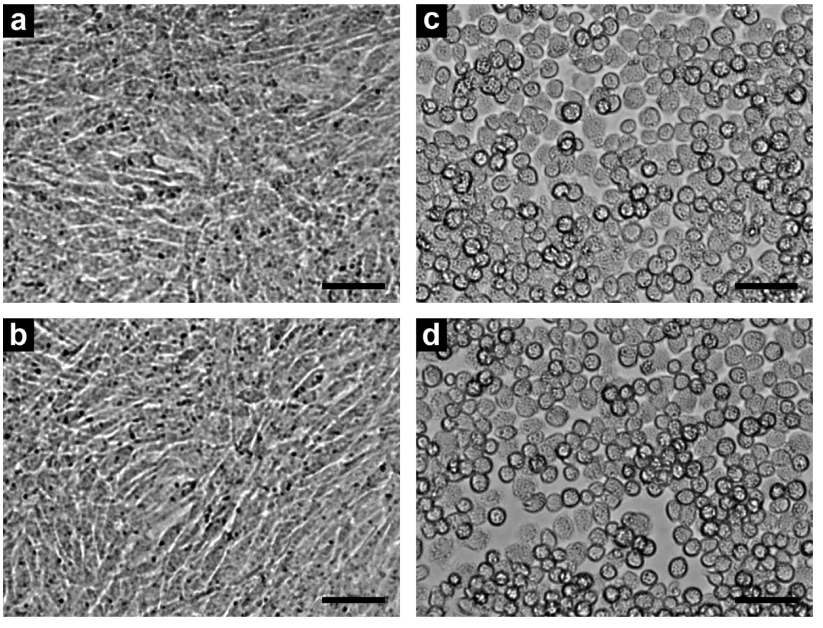


Figure 10. Morphology of NIH/3T3 cells (first column) and J774.A1 cells (second column) monitored by optical microscopy after treatment for 48 h with free SG1 at 0.1 mg mL^{-1} (line 1) and 0.3 mg mL^{-1} (line 2). Scale bar = 50 μm.

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