ATRP under Biologically Relevant Conditions: Grafting from a Protein

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Ⓢ *Supporting Information*

ABSTRACT: Atom transfer radical polymerization (ATRP) methods were developed in water-based media, to grow polymers from proteins under biologically relevant conditions. These conditions gave good control over the resulting polymers, while still preserving the protein's native structure. Several reaction parameters, such as ligand structure, halide species, and initiation mode were optimized in water and PBS buffer to yield well-defined polymers grown from bovine serum albumin (BSA), functionalized with cleavable ATRP initiators (I). The CuCl complex with ligand 2,2'-bipyridyne (bpy) provides the best conditions for the polymerization of oligo(ethylene oxide) methacrylate (OEOMA) in water at 30 °C under normal ATRP conditions ($I/CuCl/CuCl_2/bpy = 1/1/9/22$), while the CuBr/bpy complex gave better performance in PBS. Activators generated by electron transfer (AGET) ATRP gave well-controlled polymerization of OEOMA at 30 °C with the ligand tris(2-pyridylmethyl)amine (TPMA), ($I/CuBr_2/TPMA = 1/10/11$). The AGET ATRP reactions required slow feeding of a very small amount of ascorbic acid into the aqueous reaction medium or buffer. The reaction conditions developed were used to create a smart, thermoresponsive, protein–polymer hybrid.
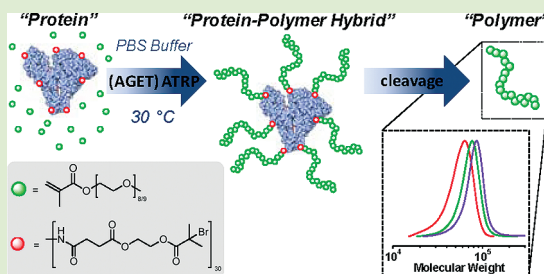
Controlled/living radical polymerizations (CRPs) allow the synthesis of polymers with predefined molecular weights, compositions, architectures, and narrow molecular weight distributions.[1] Atom transfer radical polymerization (ATRP) is among the most extensively studied and robust CRP techniques, because it is compatible with a variety of functional monomers and reaction conditions and gives polymers with high chain-end functionality.[2,3] ATRP, along with other CRP techniques, can be used to prepare biohybrids, where synthetic polymers are linked to biomolecules such as peptides, proteins, nucleic acids, and carbohydrates.[4]

Protein–polymer hybrids (PPHs) are of particular interest in academic and industrial research since they offer improved pharmacokinetics, and enhanced physical and proteolytic stability.[5] Commonly, PPHs are composed of poly(ethylene oxide) (PEO or PEG) polymer segments. Recently, however, a new generation of stimuli responsive polymers are being synthesized and conjugated to proteins to give "smart" PPHs. For instance, a smart PPH can be made by conjugating thermoresponsive polymers, such as poly(N-isopropylacrylamide) or poly(oligo(ethylene oxide) methacrylate), to a protein.[6–11]

Preparation of well-defined PPHs can be achieved by two methods: "grafting to" (g-t) and "grafting from" (g-f).[8,12,13] The g-t approach links a preformed polymer bearing a reactive chain-end to a complimentarily functionalized protein,[5,7,14–17] whereas the g-f process grows the polymer directly from an initiating site on a protein.[18,19] The g-f method leads to high yields and facile purification of the resulting hybrid,[12,13] although modification of the protein with initiating moieties

is required.[8,12] In the g-f method, ATRP initiating sites (i.e. 2-bromoisobutyrate (iBBr)) can be attached to protein either covalently[19] or through strong complexation.[20] This technique has been applied to create a variety of PPHs.[21–23] Recently, the g-f approach has been extended to activators generated by electron transfer (AGET) ATRP by utilizing ascorbic acid (AA) as reducing agent to synthesize PPHs.[18,24,25]

These advances in the g-f approach have created new opportunities to create innovative therapeutic and diagnostic systems. However, as reported in the literature,[12,26] control over the g-f process is challenging. ATRP in water has previously encountered difficulties from a very high activation rate, dissociation of halide from the X-Cu(II) deactivating species, decreased stability of Cu/ligand complexes, disproportionation of Cu(I) and hydrolysis of carbon–halogen bonds.[27,28] Typically, polymers grafted from proteins have broad molecular weight distributions (M_w/M_n), substantial tailing to low molecular weights and low initiation efficiencies. Thus, a general set of polymerization conditions for the preparation of PPHs has yet to be established. For the g-f method to become a widely accepted methodology for preparation of PPHs, these challenges must be addressed and optimized conditions must be established. Herein, ATRP methodologies are described for the synthesis of PPHs in aqueous media using the g-f approach under biologically

relevant conditions (Scheme 1). The reaction conditions were selected to maintain the protein's tertiary structure while providing a well-controlled polymerization. For protein stability, polymerizations were conducted at near ambient temperatures (30 °C), in dilute protein solutions (0.1–3.0 mg/mL), and with at least 80% water content by volume. For analytical purposes, a cleavable ATRP initiator was attached to BSA to facilitate direct gel permeation chromatography (GPC) analysis of the detached polymers (Scheme 1). This study investigates the effect of ligand, copper halide and organic cosolvent, and optimizes ATRP under the previously defined biologically relevant conditions for both normal ATRP and AGET ATRP processes. Finally, the conditions developed in this work were used to synthesize a well-defined "smart" PPH which exhibited LCST behavior.

Successful g-f ATRP requires the protein to be stable in the presence of Cu complexes.[29] The stability of green fluorescent protein (GFP) was evaluated in the presence of various copper-ligand complexes under biologically relevant polymerization conditions (1 mg/mL GFP, 10% monomer in 0.1 M PBS). Initially, three ligands (L) with different activities were selected ranging from strongly activating tris(2-pyridylmethyl)amine (TPMA), moderately activating 2,2'-bipyridine (bpy), and weakly activating N-(n-propyl)pyridylmethanimine (PI).[30]

Fluorescence measurements showed that TPMA and bpy $CuCl_2$ complexes had minimal influence on the GFP's tertiary structure, as indicated by retention of its original emission spectra (Figure S1). In contrast, both $CuCl_2$ or $CuCl_2$/PI species caused the protein to denature, as seen by a 100 fold decrease in GFP's original fluorescence intensity. Based on

these results, bpy and TPMA were selected for the development of ATRP under biologically relevant conditions.

To directly analyze the polymer grafted from BSA, the protein was modified with a cleavable ester initiator, designated as BSA-O-[iBBr]$_{30}$ (Scheme S1, Figure S2). MALDI-ToF analysis of the initiator modified BSA (BSA-O-[iBBr]$_{30}$) showed an increase in molecular mass by 9.3 kDa compared to the native BSA. This indicates that about 30 initiating sites were added to BSA and no native protein remained. The ester bond linking the initiator to the protein can be selectively cleaved after polymerization using 5% KOH (w/v) solution, without affecting the oligo(ethylene oxide) methyl ether side chains,[31] facilitating direct analysis of the g-f polymer by GPC.

Initially, normal ATRP was used to synthesize PPHs composed of a BSA protein and POEOMA polymer. Previous work illustrated that in order to achieve a successful ATRP in protic solvents a high concentration of CuX_2 deactivator is required, due to a high activation rate and partial dissociation of X-Cu(II) bond in the deactivator.[32] Based on these findings, our system was formulated with 10% of the total copper in the form of Cu(I). Initial screening experiments were performed with a PEO-macroinitiator (PEO$_{2000}$-iBBr, degree of polymerization PEO = 45; Scheme S2) and subsequently extended to the protein macroinitiator system (BSA-O-[iBBr]$_{30}$; Scheme 1). OEOMA$_{475}$ was polymerized by ATRP with a monomer volume fraction <20%, using the following molar ratios of [OEOMA$_{475}$]/[I]/[CuX]/[CuX$_2$] = 455(227)/1/1/9 and CuX to ligand of 1/22 and 1/11 for bpy and TPMA, respectively. The effect of copper halide on polymerization control was investigated by using either $CuBr$/$CuBr_2$ or $CuCl$/$CuCl_2$. Table 1 presents detailed experimental conditions and polymerization results, and Figure 1 shows the first order kinetic plot for g-f BSA-O-[iBBr]$_{30}$, number average molecular weight (M_n) versus conversion and molecular weight distribution (M_w/M_n) versus conversion. In addition, Figure 1C shows the GPC traces for normal ATRP using copper chloride salts and bpy. The Supporting Information shows similar plots for ATRP initiated by PEO$_{2000}$-iBBr (Figure S3), with both the PEO and BSA initiators essentially displaying the same behavior. In all cases, the CuX/bpy system provided better control and allowed significantly higher conversions than the CuX/TPMA system. Kinetic analysis revealed that the CuX/TPMA system reached about 5% conversion in the early stages of polymerization, after which point, the polymerization

Scheme 1. Synthesis of PPHs via (AGET) ATRP from [BSA-O-iBBr]$_{30}$ and Selective Cleavage of Polymer
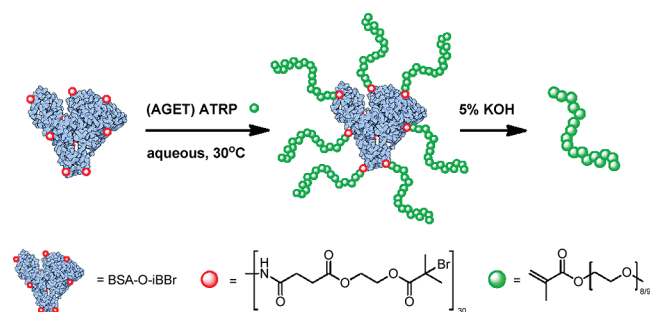
Table 1. Experimental Conditions and Results for Normal ATRP from PEO$_{2000}$-iBBr and BSA-O-[iBBr]$_{30}$[a]

	M/I/CuX/CuX$_2$/L	initiator	ligand	X	solvent	time/h	conv./%	$M_{n,theo} \times 10^{-3}$	$M_{n,GPC} \times 10^{-3}$	M_w/M_n
1	455/1/1/9/22	PEO	bpy	Br	H_2O	4	45	97	108	1.54
2	455/1/1/9/22	PEO	bpy	Cl	H_2O	4	27	58	58	1.16
3	455/1/1/9/11	PEO	TPMA	Br	H_2O	4	2	4	12	1.27
4	455/1/1/9/11	PEO	TPMA	Cl	H_2O	4	2	4	18	1.22
5	227/1/1/9/22	BSA	bpy	Br	H_2O	3.5	75	81	73	1.54
6	227/1/1/9/22	BSA	bpy	Cl	H_2O	3.5	70	75	70	1.16
7	227/1/1/9/11	BSA	TPMA	Br	H_2O	4	5	5	40	1.10
8	227/1/1/9/11	BSA	TPMA	Cl	H_2O	4	2	2	35	1.16
9	227/1/1/9/22	BSA	bpy	Cl	DMSO/H_2O	3.5	75	81	84	1.22
10	200/1/1/9/22	BSA	bpy	Cl	DMSO/H_2O	3.5	82	40	58	1.22
11	227/1/1/9/22	PEO	bpy	Cl	PBS	3	6	6	10	1.19
12	227/1/1/9/22	PEO	bpy	Br	PBS	3	33	36	28	1.17
13	227/1/1/9/22	BSA	bpy	Br	PBS	3	40	43	50	1.26

[a][I] = 1 mM; 30 °C; entries 1–4, 9: 20% [M] (v/v); entries 5–8, 10–13: 10% [M] (v/v); M = OEOMA$_{475}$, except entry 10: [OEOMA$_{300}$]/[MEO$_2$MA] = 1/1.
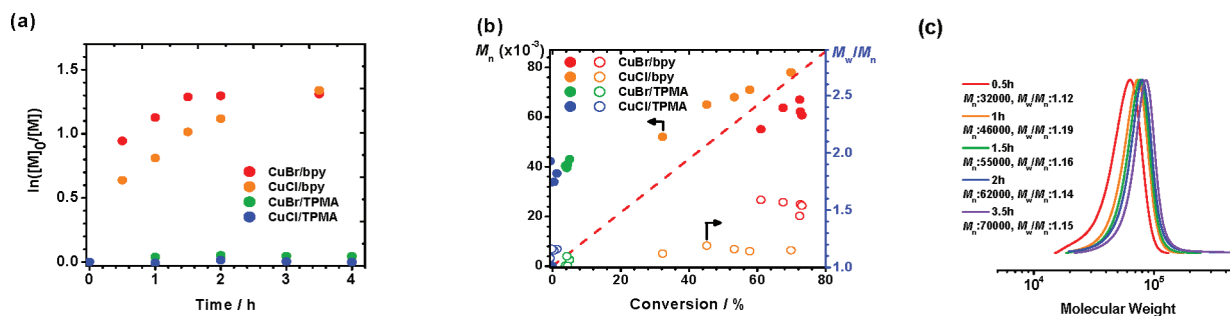
Figure 1. Effect of ligand (L = bpy or TPMA) and halide (X = Br or Cl) on ATRP of OEOMA$_{475}$ from BSA-O-[iBBr]$_{30}$ at 30 °C (Table 1, entries 5−8). (A) First order kinetic plot and (B) M_n and M_w/M_n vs conversion plot; (C) GPC traces for CuCl/CuCl$_2$/bpy (Table 1, entry 6). [OEOMA$_{475}$]$_0$ = 0.21 M; [OEOMA$_{475}$]/[I]/[CuX]/[CuX$_2$]/[L] = 227/1/1/9/11 ([L]/[TPMA] = 2[bpy]).

Table 2. Experimental Conditions and Results for AGET ATRP from PEO$_{2000}$-iBBr and BSA-O-[iBBr]$_{30}$[a]

	M/I/CuBr$_2$/L/AA	initiator	ligand	solvent	time/h	conv./%	$M_{n,theo} \times 10^{-3}$	$M_{n,GPC} \times 10^{-3}$	M_w/M_n
1	455/1/10/22/0.1	PEO	bpy	H$_2$O	6	20	43	25	1.30
2	455/1/10/11/0.1	PEO	TPMA	H$_2$O	6	15	32	30	1.09
3	455/1/10/11/0.01	PEO	TPMA	H$_2$O	6	5	11	15	1.09
4	455/1/10/11/0.03[b]	PEO	TPMA	H$_2$O	1	12	26	27	1.10
5	227/1/10/11/0.1[c]	PEO	TPMA	H$_2$O	1.5	78	84	100	1.16
6	227/1/10/11/0.1	BSA	TPMA	H$_2$O	4	5	5	30	1.10
7	227/1/10/11/0.1[c]	BSA	TPMA	H$_2$O	4	88	95	82	1.08
8	227/1/10/11/0.1[c]	PEO	TPMA	PBS	4	71	77	93	1.12
9	227/1/10/11/0.1[c]	BSA	TPMA	PBS	4	75	81	83	1.19

[a][I] = 1 mM; 30 °C; entries 1−4: 20% [M] (v/v); entries 5−9: 10% [M] (v/v); water. [b]AA was added stepwise. [c]AA was fed to the reaction mixture at the rate 8 nmol/min.

stopped. These results suggest that the TPMA based catalyst is too active, leading to a very high concentration of radicals and significant termination in the early stages of polymerization. In addition to the ligand, the halide species had a significant impact on polymerization behavior, especially for the CuX/bpy systems. Polymerization kinetics catalyzed by CuCl/bpy showed a more linear semilogarithmic plot, and narrower polymer distributions compared to the CuBr/bpy system (Figures 1 and S4). This behavior can be partially attributed to the increased activity of the Br-based initiators/chain ends compared to their corresponding Cl analogs, leading to a larger fraction of terminated chains.[30] Therefore, the optimal condition and results for g-f using normal ATRP are shown in Table 1 (entry 6) and presented in Figure 1 (orange data series and GPC traces).

AGET ATRP was next investigated for the synthesis of PPHs. In this AGET ATRP approach, the CuX/L activator was generated in situ from oxidatively stable CuX$_2$/L using ascorbic acid (AA).[33] The reaction conditions for AGET ATRP in water from PEO$_{2000}$-iBBr and BSA-O-[iBBr]$_{30}$ with AA as a reducing agent are presented in Table 2. In all experiments, the amount of added AA was from 0.1 to 2.0% of the total amount of CuBr$_2$. A single injection of reducing agent in AGET ATRP showed results similar to that of the TPMA system using normal ATRP. In particular, there was a small monomer conversion in the initial stages, and no further conversion after this initial period (Figures 2 and S5). Despite that monomer conversion was low, the polymer synthesized using TPMA had a lower M_w/M_n value than polymers obtained using bpy (Table 2, entries 1 and 2). Typically, AGET ATRP is performed with the entire charge of reducing agent injected once at the beginning of polymerization. However, results in this work with PEO$_{2000}$-iBBr (Figure S6 and S7) and, as in a previous report,[34]

suggest that continuous and slow feeding of AA in AGET ATRP should lead to lower radical concentrations, diminished termination and promote continued monomer conversion. Figure 2 illustrates that slow addition of 1 mol % of AA (vs CuBr$_2$) leads to 88% conversion in 4 h, compared to 5% conversion when the same amount of AA was added in a single charge. The polymer formed by slowly feeding AA had high molecular weight and a narrow molecular weight distribution (M_w/M_n = 1.08; Table 2, Figure 2). Because one molecule of ascorbic acid provides two electrons,[35] the total amount of AA added after 4 h of polymerization corresponds to no more than 2% of generated Cu(I). Thus, AGET ATRP in water can be used to prepare well-defined PPHs using a very small amount of reducing agent added over an extended period of time.

Polymerization conditions were also investigated for the g-f reaction in phosphate-buffered saline (PBS) solution. PBS (pH = 7.4) is a widely utilized protein buffer[36] and served as the reaction medium for the g-f polymerizations. ATRP in PBS is challenging for several reasons. First, copper and phosphate ions can form insoluble Cu$_3$(PO$_4$)$_2$, and second, chloride anions within the buffer can displace ligands from their copper centers producing a relatively inactive catalyst. To determine optimal conditions for a well-controlled polymerization in PBS, OEOMA$_{475}$ was polymerized in PBS using both normal ATRP and AGET ATRP. Experimental conditions and polymerization results are summarized in Tables 1 (entries 11−13) and 2 (entries 8−9) for normal and AGET ATRP, respectively.

The CuCl/CuCl$_2$/bpy catalyzed polymerization in PBS resulted in minimal conversion of monomer after 3 h (Table 1, entry 12), while the CuBr/CuBr$_2$/bpy catalyzed polymerization in PBS (Table 1, entry 11) was approximately 3 times slower than the reaction in water (Table 1, entry 1). Furthermore, the polymerization was well controlled up to
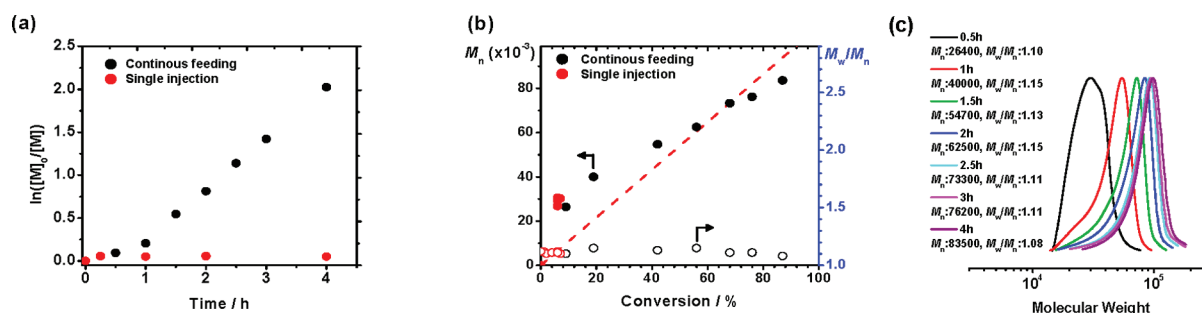
Figure 2. Effect of single injection or continuous feeding of reducing agent on AGET ATRP of $OEOMA_{475}$ g-f BSA-O-$[iBBr]_{30}$ at 30 °C (Table 2, entries 6 and 7,). (A) First order kinetic plot and (B) M_n and M_w/M_n vs conversion plot; (C) GPC traces with continuous feeding of AA (Table 2, entry 7). Polymerizations conducted with $[OEOMA_{475}]_0 = 0.21$ M and $[OEOMA_{475}]/[I]/[TPMA]/[CuBr_2] = 227/1/11/10$. Feeding rate of AA was 8 nmol/min.
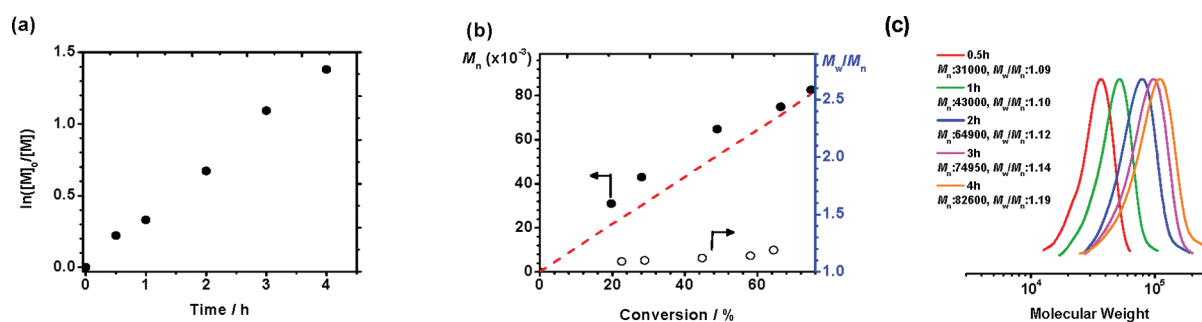
Figure 3. AGET ATRP of $OEOMA_{475}$ g-f BSA-O-$[iBBr]_{30}$ at 30 °C in PBS. (A) First order kinetic plot and (B) M_n and M_w/M_n vs conversion plot; (C) Corresponding GPC traces. $[OEOMA_{475}]_0 = 0.21$ M; $[OEOMA_{475}]/[I]/[CuBr_2]/[TPMA] = 227/1/10/11$. Feeding rate of AA was 8 nmol/min.

30%, after which time, the semilogarithmic plot became noticeably curved (Figures S8 and S9). Polymers grown from BSA-O-$[iBBr]_{30}$ in the presence of CuBr/CuBr$_2$/bpy had relatively narrow molecular weight distributions. When AGET ATRP with TPMA ligand was used with slow feeding of AA, there was a linear increase in the first-order kinetic plot up to high monomer conversion for both PEO and BSA macro-initiators (Figures 3A and S10). The feeding rate was the same in both experiments performed in water and PBS. Figure 3B illustrates that AGET ATRP gives a nearly linear increase in molecular weight with conversion while maintaining M_w/M_n below 1.2. The GPC traces in Figure 3C show monomodal distributions and a gradual shift to higher molecular weights with time. Thus, (AGET) ATRP can be used to create well-controlled PPHs in PBS buffered solutions.

Finally, polymerizations were performed with 10% (v/v) dimethyl sulfoxide (DMSO). The addition of an organic solvent provides a medium suitable for monomers insoluble in purely aqueous systems (e.g., MEO_2MA). Furthermore, DMSO is a useful solvent in proteomics,[37] because a concentration below 10% (v/v) does not denature proteins.[37] Reaction conditions are summarized in the Table 1 (entries 6 and 9), with first-order kinetic plots, molecular weight evolutions with conversion, and molecular weight distribution versus conversion shown in the SI (Figure S11). The rate of polymerization, molecular weight values, and M_w/M_n values were essentially the same as those obtained without DMSO. Finally, a well-defined thermoresponsive copolymer of MEO_2MA and $OEOMA_{300}$ was grafted from BSA-O-$[iBBr]_{30}$ (Table 1, entry 10) by normal ATRP under these conditions. The polymer grafted from the protein had a $M_{n(GPC)} = 58000$ and $M_w/M_n = 1.22$. This PPH had an LCST of 52 °C (Figure S12), as seen by

a reversible change of the PPH's hydrodynamic diameter from 40 nm to 5 μm, above and below its LCST, respectively.

In conclusion, this letter shows how well-defined polymers can be grafted from proteins by (AGET) ATRP under biologically relevant conditions. These conditions are designed both to maintain protein stability throughout the polymerization and grow polymers with narrow molecular weight distributions. Biologically relevant conditions have been defined as near ambient temperatures (ca. 30 °C), low initiator concentrations (<2 mM), and low monomer and cosolvent concentrations (total organic content should not exceed 20% of the total reaction volume). Furthermore, the catalyst selected must bind to copper sufficiently strongly to prevent protein denaturation. When conducting traditional ATRP, the optimal catalyst was CuX/CuX_2/bpy (1/9/22), where X is either Cl or Br. The optimal halide depends upon the reaction media selected: in pure water Cl is preferred, while in PBS, Br is required to maintain an acceptable polymerization rate. AGET ATRP with slow feeding of ascorbic acid allows for strongly activating TPMA based catalysts to be used at very low ratios of copper(I) to copper(II). Moreover, AGET ATRP with slow feeding of AA gives a rapid reaction and well-controlled polymers in both pure water and PBS. Finally, the use of 10% of an organic solvent (DMSO) expands range of available monomers, giving access to smart biohybrid materials.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials, instrumentation, experimental conditions, and experimental results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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