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Polymer−Protein Conjugation in Ionic Liquids

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PEGylation chemistry is used extensively to increase the size of proteins, reduce protein clearance rate *in vivo*, shield proteins from enzymatic degradation, and improve their pharmacokinetic (PK) profile.[1] Advantages of PEGylation have led to therapeutics in use today, such as PEG-Intron and Pegasys for the treatment of hepatitis C.[2] Phosphorylcholine-containing polymers, such as poly(methacryloyloxyethylphosphorylcholine) (polyMPC), are very hydrophilic and have been suggested as alternatives to PEG for protein conjugation. For example, polyMPC has been conjugated to the therapeutic proteins interferon, erythropoeiten (EPO), and granulocyte-colony stimulating factor (G-CSF), using end-functional derivatives prepared by atom transfer radical polymerization (ATRP).[3,4] Such conjugates were seen to possess *in vivo* PK profiles superior to PEGylated proteins, likely due to the extensive water structure associated with the zwitterionic backbone.[5]

The success of protein PEGylation stems largely from the commercial availability of useful PEG derivatives, such as *N*-hydroxysuccinimidyl ester (NHS)-terminated PEG for amidation of amine-containing residues on the protein structure. We prepared analogous NHS-functionalized polyMPC by ATRP of MPC from an appropriate NHS-containing initiator[3] and in some cases have experienced low protein conjugation efficiency, likely due to competitive hydrolysis of the NHS chain-end during attempted conjugation. The extremely hydrophilic nature of polyMPC makes such labile end-groups susceptible to hydrolysis if sufficient care is not taken to prevent loss of the chain-end during polymerization, workup, and/or storage, and the resultant nonfunctional chain ends preclude protein conjugation. Moreover, the strictly hydrophilic nature of polyMPC (as distinct from the amphiphilicity of PEG) restricts polymerization solvent choices to water, methanol, and their mixtures with polar solvents like DMSO. Taking these factors together, we recently turned to ionic liquids (ILs)[6] as potential alternative solvents for polyMPC preparation and conjugation and report here a unique advantage of ILs for polyMPC preparation and conjugation to proteins. PolyMPC preparation and protein conjugation proved efficient in ILs, as the IL provides a stable solution environment for the NHS chain ends and high yields in both the polymer chain-growth and subsequent protein conjugation steps.

As the ATRP mechanism functions effectively in ILs for styrene and methyl methacrylate,[7,8] we decided to attempt ATRP of the zwitterionic MPC in ILs, initiating from NHS-carbonate **1**, shown in Scheme 1. We found that, in ILs, NHS-terminated polyMPC **3** could be prepared with little-to-no loss of the NHS end group. Room temperature ILs are particularly relevant to polymer synthesis,[9,10] and as such we tested each of the possible cation−anion combinations shown in Scheme 1 (i.e., 1-butyl-3-methylimidazolium (BMIM) and 1-ethyl-3-methylimidazolium (EMIM) cations and tetrafluoroborate (BF_4) and trifluoromethanesulfonate (CF_3SO_3) anions).

The results of room temperature ATRP of MPC in ILs are summarized in Table 1. We prepared polyMPC successfully in three different ILs: BMIM·BF_4, EMIM·CF_3SO_3, and BMIM·CF_3SO_3 (entries 1−3). Monomer conversions of 80% or greater (based on ^{1}H NMR spectroscopy) were achieved in less than 6 h, giving number-average molecular weights (M_n) of ∼8 kDa. Use of an acetonitrile solution of CuBr catalyst and bipyridine (bpy) ligand proved optimal for catalyst and ligand solubility and for maintaining a reasonable reaction mixture viscosity. Following the polymerization, the acetonitrile was removed under vacuum, while the IL was removed by precipitation in dry acetone, and residual monomer and catalyst were removed by passage through a short plug of silica gel. An exemplary GPC trace of polyMPC, as prepared in BMIM·CF_3SO_3 (Figure 1A), shows a narrow polydipsersity index (PDI) of 1.3. All of the hydrophilic ILs tested were seen to solubilize both MPC monomer and polyMPC at room temperature, providing a convenient nonaqueous solution environment that is inert to the NHS end groups and effective for protein−polymer conjugation.

ATRP of MPC in EMIM·BF_4 proceeded rapidly, resulting in gelation of the reaction mixture in minutes. Molecular weight characterization revealed the polyMPC to be of higher-than-targeted molecular weight and PDI (entry 4, Table 1). Interestingly, ATRP in EMIM·BF_4 in the absence of added bpy (entry 5) also led to rapid polymerization, suggesting the potential for ILs to serve as the copper coordinating species in ATRP chemistry, though we note that the polymerization is not as well-controlled under these conditions, leading to high molecular weight polymers with high polydispersity (∼1.9). We also extended this concept to poly(ethylene glycol) (PEG)-substituted methacrylate monomers (e.g., PEG-MA_{300} in entry 6 of Table 1). PEG-MA monomers polymerized cleanly by ATRP in EMIM·BF_4, even without added ligand. In the PEG-MA_{300} case, monomer conversion followed the trend characteristic of a living polymerization, reaching over 90% conversion in 5 h and giving estimated molecular weights in agreement with those expected for the chosen monomer-to-initiator ratio (Figure 1B). This result suggests that EMIM·BF_4 can act as both solvent and binding ligand for copper and potentially bind with the zwitterionic PC groups, leading to accelerated chain propagation and a nonliving behavior of the system.

We note that the zwitterionic polyMPC structures can participate in associative interactions with the IL. For example, for polyMPC preparation in EMIM·BF_4, the IL remained with the polyMPC structure after precipitation into acetone. This was seen in the ^{1}H NMR spectra of the polyMPC products, showing a nearly equimolar ratio of EMIM and MPC units. Moreover, ^{19}F NMR spectroscopy confirmed the presence of the BF_4 counterion. PolyMPC-IL complexation was also observed upon mixing pure polyMPC with EMIM·BF_4, though this IL−polymer coordination had little effect on the GPC-estimated molecular weight compared to the pure polymer material.

The presence of the NHS carbonate chain end on these polyMPC structures was confirmed by ^{1}H NMR spectroscopy in CD_3OD (Figure 2). The signal at 2.81 ppm, corresponding to the NHS methylene protons bound to polyMPC, shifts to

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Scheme 1. One-Pot Polymerization and Protein Conjugation in ILs

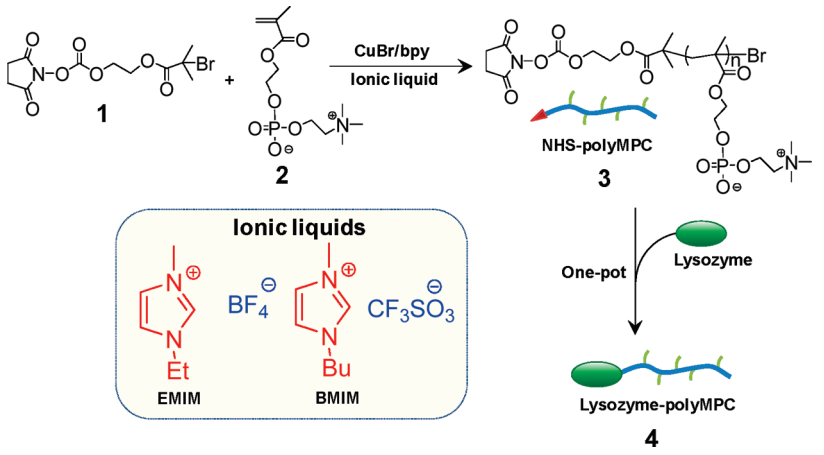

Table 1. PolyMPC Structures Prepared in Different Ionic Liquids (ILs)

entry	monomer	IL	ligand	target M_n (kDa)[a]	conversion[b] (%)	GPC M_n (kDa)	PDI
1	MPC	EMIM·CF_3SO_3	bpy	11	78	8	1.4
2	MPC	BMIM·BF_4	bpy	9	75	6	1.4
3	MPC	BMIM· CF_3SO_3	bpy	11	80	8	1.3
4	MPC	EMIM·BF_4	bpy	6	98	53	2.9
5	MPC	EMIM·BF_4		6	90	100	1.9
6	PEG-MA_{300}	EMIM·BF_4		11	96	11	1.6

[a] Based on monomer-to-initiator ratios. [b] By ^{1}H NMR.

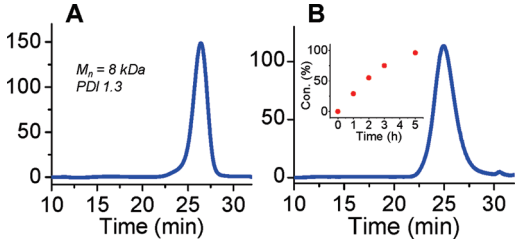

Figure 1. (A) Aqueous GPC trace of NHS-terminated polyMPC prepared in IL (Entry 3 in Table 1). (B) Aqueous GPC trace of poly(PEG-MA_{300}) prepared in EMIM·BF_4 without adding ligand (M_n 11 kDa) (the inset plots monomer conversion on the *y*-axis vs time on the *x*-axis).

2.59 ppm in the released NHS. Purified NHS-terminated polyMPC **3** (20 equiv) was then used in protein conjugation chemistry with lysozyme (1 equiv) in sodium borate buffer at pH 9.0. Successful conjugation was noted by the SEC-HPLC trace of Figure 3, showing no unreacted lysozyme, which would elute at 11.5 min. Complete conversion of free lysozyme to the corresponding conjugate was characterized by cation-exchange fast protein liquid chromatography (FPLC), and high conjugation efficiency was enabled by the presence of the NHS-ester chain ends.

ILs have been utilized recently as dissolution media for proteins and enzyme-based processes,[11] and room temperature protein stabilization in ILs has been demonstrated.[12,13] Here, we demonstrate that polyMPC−protein conjugation can be performed efficiently in ILs in a one-pot fashion immediately following MPC polymerization. Pure lysozyme dissolved slowly in the ILs used here, but conjugation proceeded effectively when lysozyme was added as a buffered solution (i.e., sodium borate buffer at pH 9.0) to the MPC polymerization mixture (i.e., entry 1 of Table 1) in EMIM·CF_3SO_3 at room temperature. At a reaction time of 2 h, SEC-HPLC analysis revealed exceptionally efficient conjugation with no free lysozyme detected (Figure 3). Little aggregation (< 5%) was seen (eluting at ~5.5 min on SEC-HPLC) in these conjugate samples. For comparison and as a control experiment, we note that if the IL/polymer solution is

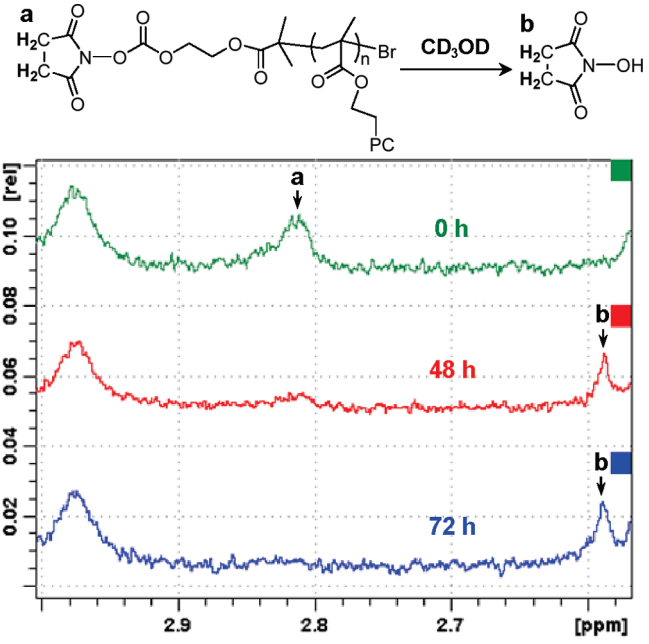

Figure 2. ^{1}H NMR spectra in CD_3OD showing a shift of the NHS methylene protons before and after release from the polyMPC chain end after 0 h (top), 48 h (middle), and 72 h (bottom).

pretreated with ethanolamine, the polymer is deactivated, and no conjugation is observed.

In summary, we have described a unique advantage of ILs for protein−polymer conjugation, both as a two-step and one-pot process, where the polymerization and conjugation chemistry both proceed efficiently in the IL medium. The nonaqueous polar environment provided by the IL is key to the process, providing access to, to our knowledge, the first study of protein conjugation in IL solution, with potential for extension to a breadth of protein conjugation chemistries.

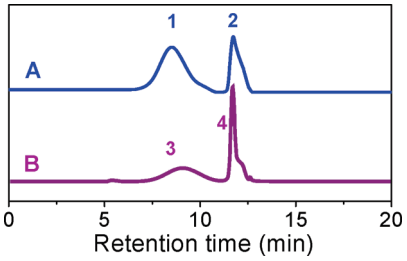

Figure 3. SEC-HPLC traces of the protein conjugation reaction mixtures. (A) Two-pot conjugation; peak 1: lysozyme−polyMPC conjugate, peak 2: NHS. (B) One-pot conjugation; peak 3: lysozyme−polyMPC conjugate, peak 4: coelution of NHS and IL.

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Supporting Information Available: Experimental details for the synthesis and characterization of the initiator, polymers, and conjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

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