

SYNTHESIS OF POLYMERIC MIMICS OF ICE-BINDING PROTEINS

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ABSTRACT

Cobalt-mediated radical polymerization (CMRP) enables the preparation of both short and long polymers from acrylic and vinyl ester monomers with low dispersity. Here we describe the synthesis, purification, and characterization of polymeric mimics of ice-binding proteins based on the water-soluble polymer poly(vinyl alcohol) by CMRP. Block copolymers of poly(vinyl alcohol) and poly(acrylic acid) were prepared from the precursor copolymers poly(vinyl acetate)-*b*-poly(acrylonitrile) upon hydrolysis. Copolymers comprising up to hundreds of monomers and dispersities $M_w/M_n < 1.3$ were produced by this method.

KEYWORDS

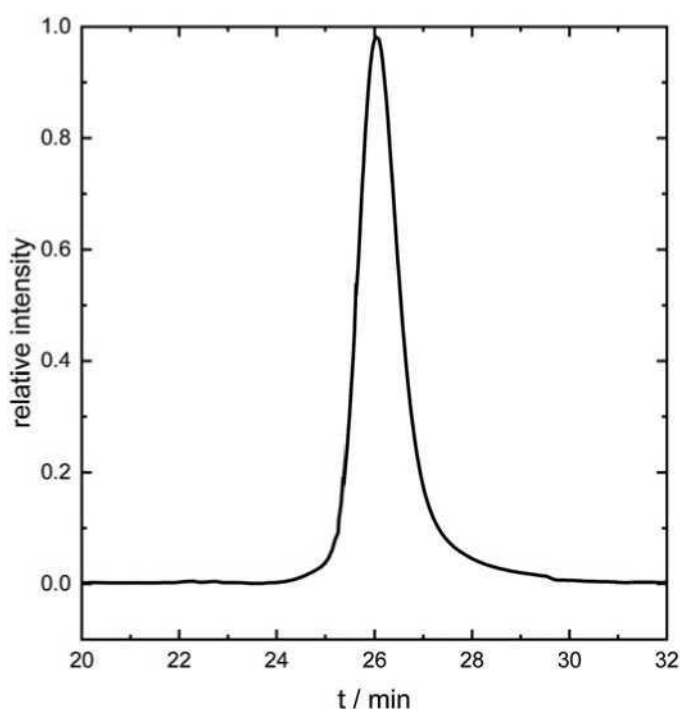
Cobalt-mediated radical polymerization - Controlled radical polymerization - Poly(vinyl alcohol) - Poly(vinyl alcohol)-*b*-poly(acrylic acid) - Polymeric mimic of ice-binding proteins - Polymeric antifreeze - Size exclusion chromatography - ^1H NMR spectroscopy

Introduction

Controlled radical polymerization (CRP) techniques offer access to a broad range of polymers customized in composition, dimensions, and chain architecture. Since the discovery of CRP in 1993 [1], various methods have been established, including nitroxide-mediated radical polymerization (NMP) [2, 3], atom transfer radical polymerization (ATRP) [4], radical addition–fragmentation chain transfer (RAFT) [5], and cobalt-mediated radical polymerization (CMRP) [6, 7]. Most polymeric ice-binding protein (IBP) mimics are based on the water-soluble polymer poly(vinyl alcohol) (PVOH), which is typically prepared by CMRP [8] or RAFT polymerization [9, 10] of vinyl acetate followed by hydrolysis of the prepared poly(vinyl acetate). Above a sufficiently high molecular weight, PVOH is able to inhibit ice recrystallization, i.e., the polymer displays ice recrystallization inhibition (IRI) activity. PVOH is the most IRI active polymer known to date with an inhibitory concentration c_i of 0.3–0.4 mM vinyl alcohol (corresponding to 0.013–0.018 g L⁻¹) above which ice crystal recrystallization

rates drop markedly. Compared to natural IBPs, PVOH is moderately active. Interestingly, the IRI activity of PVOH is little affected by adjustments in the composition of the polymer, as long as sufficiently long stretches of vinyl alcohol monomers remain included.

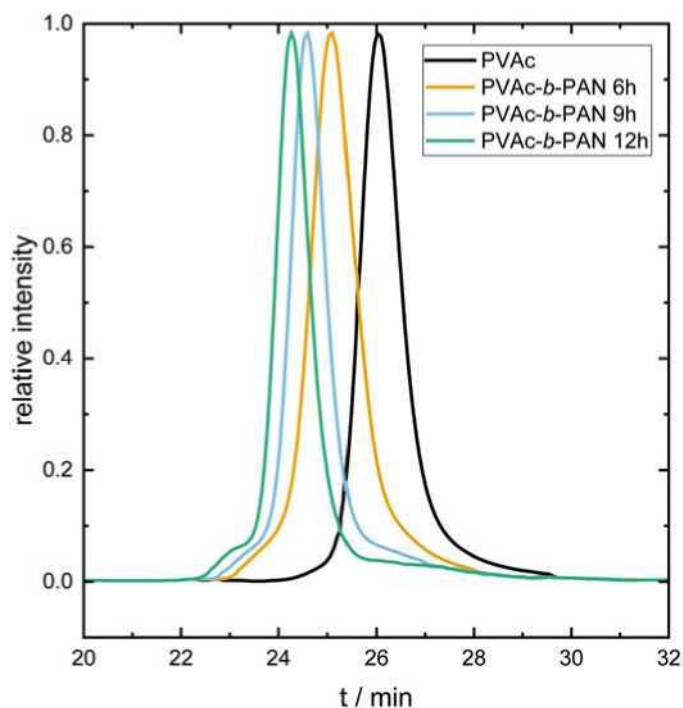
Fig. 1 SEC chromatogram of the PVAc macroinitiator in DMF with 0.025 M LiBr



This offers ample room to customize polymer composition, architecture, and solution behavior to develop IRI active materials with interesting and diverse (auxiliary) properties. Here we describe the synthesis, purification, and characterization of the block copolymer poly(vinyl alcohol)-*b*-poly(acrylic acid) (PVOH-*b*-PAA), which has been used to prepare IRI active polymer micelles [8]. The procedure for the preparation of PVOH-*b*-PAA has been reported previously in [11]. PVOH-containing polymers varying in composition and architecture have been achieved in a similar fashion [12–14]. Polymers with hundreds of monomers and dispersities $M_w/M_n < 1.3$ were prepared as demonstrated by size exclusion chromatography (SEC) (see Figs. 1 and 2) and ^1H NMR spectroscopy (see Figs. 3 and 4).

Materials

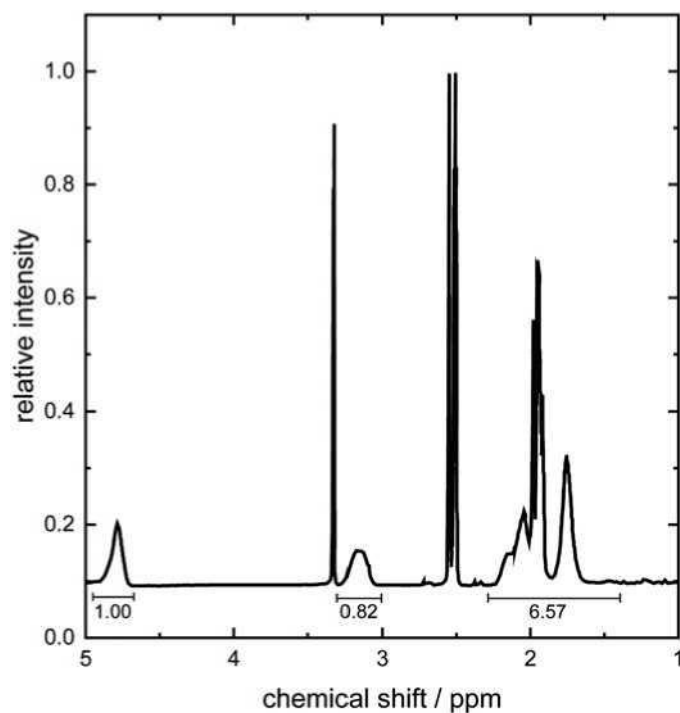
Fig. 2 SEC chromatogram of the PVAc-*b*-PAN precursors, after 6 (yellow), 9 (blue), and 12 h (green) of polymerization, compared to the trace of the PVAc macroinitiator (black), in DMF with 0.025 M LiBr



1. Vinyl acetate (>99%, Aldrich). Dry over calcium hydride. Degas by several freeze–thawing cycles. Distill under reduced pressure. Store under argon.
2. Acrylonitrile (>99%, Aldrich). Dry over calcium hydride. Degas by several freeze–thawing cycles. Distill under reduced pressure. Store under argon.
3. 2,2'-Azobis(4-methoxy-2,4-dimethylvaleronitrile) (V-70) (Wako).
4. Cobalt(II) acetylacetonate ($\text{Co}(\text{acac})_2$) (>98%, Merck).
5. 2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO) (98%, Aldrich).
6. 500 mL flask.
7. 5 L flask.
8. 3 L flask.
9. Plastic syringe.

10. Nitrogen gas.
11. Tetrahydrofuran (THF).
12. Dimethylformamide (DMF) with 0.025 M LiBr.
13. Methanol.
14. Ethanol (p.a.).
15. DMSO-d₆.
16. KOH.
17. Deionized water.

Fig. 3 ¹H NMR spectrum of the precursor PVAc-*b*-PAN in DMSO-d₆. δ (ppm): 3.14 (2H, CH₂-CH-CN of PAN), 4.78 (2H, CH₂-CH-OCOCH₃ of PVAc)



18. Spectra/Pore membrane (cutoff: 6000–8000 Da).
19. Polystyrene standards ranging from 225 to 580×10^4 g/mol for SEC calibration.
20. Waters 600 liquid chromatograph equipped with a 410 refractive index detector and Styragel HR columns.

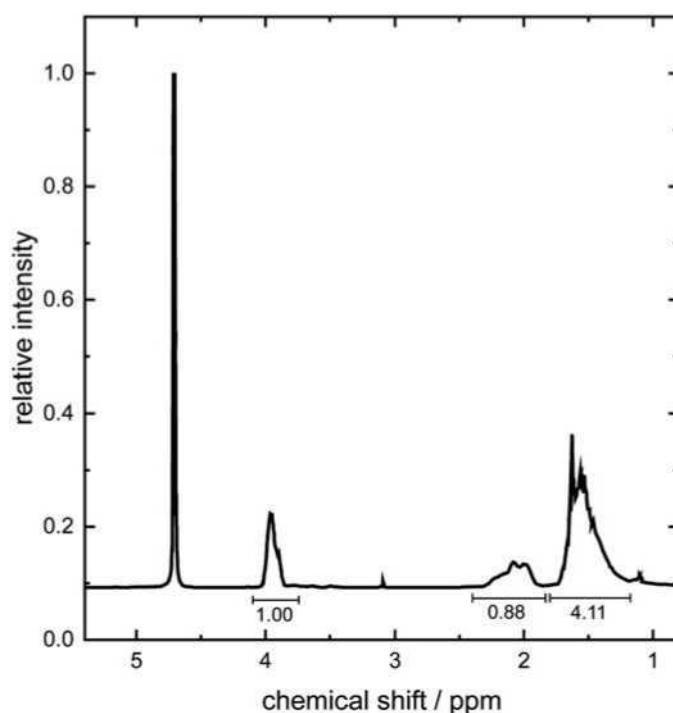
21. 250 MHz Bruker spectrometer.

Methods

SYNTHESIS, PURIFICATION, AND CHARACTERIZATION OF THE POLY(VINYL ACETATE)-B- POLY(ACRYLONITRILE) PRECURSOR PVAC-B-PAN

1. Add $\text{Co}(\text{acac})_2$ (1.03 g, 4.02 mmol) and V-70 (4.01 g, 13.0 mmol) in a 500 mL flask.
2. Degas the mixture three times by vacuum–nitrogen cycles.
3. Add vinyl acetate (150 mL, 1.62 mol) with a syringe under nitrogen.
4. Stir the purple mixture at 30 °C for 26 h.
5. After 26 h, withdraw a sample for characterization: evaluate the monomer conversion gravimetrically, and measure the molar mass and dispersity of the PVAc macroinitiator by SEC analysis in DMF with 0.025 M LiBr (see Fig. 1).

Fig. 4 ^1H NMR spectrum of PVOH-*b*-PAA in D_2O . δ (ppm): 3.9 (1H, $\underline{\text{C}}\text{H-OH}$ of PVOH), 3.09 (3H, end group), 2.05 (1H, $\underline{\text{C}}\text{H-COO-K}^+$ of PAA), 1.80–1.0 (2H, $\underline{\text{C}}\text{H}_2\text{-CHOH}$ of PVOH and 2H, $\underline{\text{C}}\text{H}_2\text{-CH-COO-K}^+$ of PAA)



6. Evaporate the residual monomer under reduced pressure at room temperature.
7. Store the pink poly(vinyl acetate) macroinitiator under inert atmosphere.
8. Cool the flask to 0 °C in an ice bath and add 30 mL of dry DMSO.
9. Add 25 mL of acrylonitrile (AN).
10. Stir the reaction medium at 0 °C for about 12 h to polymerize AN. Remove 10 mL of the reaction mixture after 6 h and another 10 mL after 9 h. During AN polymerization, the medium became viscous but remained homogeneous, and the color changed from pink to gray black.
11. Add the two 10 mL aliquots and the final reaction mixture to a solution of TEMPO (50 mg in 5 mL of DMSO).
12. Disperse the copolymer in a methanol/water (20/80) mixture from which it will precipitate. Filter the copolymer using a paper filter and wash it with more MeOH/H₂O. Dry the copolymer under vacuum at 80 °C.
13. Collect the copolymer (~9 g) for analysis by SEC in DMF with 0.025 M LiBr (see Fig. 2) and ¹H NMR in DMSO-d₆ (see Fig. 3).
14. Dissolve 5 wt% copolymer for ¹H NMR analysis (250 MHz, 298 K) in DMSO-d₆ (see Fig. 3). Compare the intensities of the signals corresponding to CH₂-CH-CN of PAN (at 3.14 ppm) and CH₂-CH-OCOCH₃ of PVAc (at 4.78 ppm).
15. Calculate the molar mass of the PAN block from the ¹H NMR data of the diblock in DMSO-d₆ and M_n of the PVAc macro-initiator determined by SEC in THF. Having normalized the intensity for a single proton of PVAc at 4.78 ppm, the peak at 3.14 ppm corresponds to a single proton of PAN, with an area of 0.82. Hence:

$$M_{n,PAN} = M_{n,PVAc} \cdot \frac{M_{0,PAN}}{M_{0,PVAc}} \cdot \frac{A_{3.14\text{ppm}}}{A_{4.78\text{ppm}}}$$

SYNTHESIS, PURIFICATION, AND CHARACTERIZATION OF POLY(VINYL ALCOHOL)-B- POLY(ACRYL ACID) (PVOH-B-PAA)

1. Dissolve the precursor PVAc-b-PAN (30 g) in 600 mL ethanol in a 5 L flask.
2. Prepare an aqueous solution of potassium hydroxide: dissolve 120 g of KOH in 2700 mL of water in a 3 L flask.

3. Add the aqueous solution of potassium hydroxide to the precursor solution in ethanol.
4. Stir the mixture at 75 °C for 48 h under reflux. Over the course of hours, the copolymer was sufficiently hydrolyzed to slowly dissolve in the reaction medium to form a homogeneous solution.
5. Remove ethanol under vacuum.
6. Dialyze the aqueous copolymer solution for 48 h against water through a Spectra/Pore membrane (cutoff: 6000–8000 Da).
7. Lyophilize the copolymer.
8. Recover the copolymer as a white powder.
9. Dissolve 5 wt% copolymer in D₂O for ¹H NMR analysis (250 MHz, 298 K) (see Fig. 4).
10. Calculate the molar mass of the PVOH block from the ¹H NMR data of the diblock in D₂O.

$$M_{n,PVOH} = 3 \cdot M_{0,PVAc} \cdot \frac{A_{3.90\text{ppm}}}{A_{3.09\text{ppm}}}$$

The molar ratio of the PVOH/PAA blocks calculated from the ¹H NMR spectrum of PVOH-*b*-PAA was very similar to the molar ratio of the PVAc/PAN blocks of the PVAc-*b*-PAN precursor.

$$M_{n,PAA} = M_{n,PVOH} \cdot \frac{M_{0,PAA}}{M_{0,PVOH}} \cdot \frac{A_{2.05\text{ppm}}}{A_{3.90\text{ppm}}}$$

SIZE EXCLUSION CHROMATOGRAPHY (SEC)

1. Perform size exclusion chromatography (SEC) analysis on a Waters 600 liquid chromatograph equipped with a 410 refractive index detector and Styragel HR columns. Use polystyrene (PS) standards with molar masses ranging from 225 to 580 × 10⁴ g/mol for calibration.
2. For SEC analysis of the molar mass and dispersity of the PVAc macroinitiator and PVAc-*b*-PAN precursor in DMF with 0.025 M LiBr (see Fig. 1): use a flow rate of 1 mL min⁻¹ and a temperature of 55 °C. Use Styragel HR columns (HR1, 100–5000; HR3, 500–30,000; HR4, 5000–500,000; HR5, 2000–40,000,000). Use PS standards for calibration (see Note 1).

¹H NMR SPECTROSCOPY

1. Record ¹H NMR spectra on a 250 MHz Bruker spectrometer running at 298 K.
2. For ¹H NMR analysis of the PVAc macroinitiator: Dissolve 5 wt% PVAc in CDCl₃ and record 16 scans with $D_1 = 2$ s.
3. For ¹H NMR analysis of the precursor PVAc-*b*-PAN: Dissolve 5 wt% of the precursor in DMSO-*d*₆ (see Fig. 3) and record 32 scans with $D_1 = 5$ s.
4. For ¹H NMR analysis of the copolymer PVOH-*b*-PAA: Dissolve 5 wt% of the copolymer in D₂O (see Fig. 4) and record 32 scans with $D_1 = 5$ s.

Notes

1. The molar mass determination of the PVAc macroinitiator by SEC analysis using PS standards and ¹H NMR in CDCl₃ was in good agreement whenever the -OCH₃ protons of the initiator at $\delta = 3.13$ ppm could be observed and compared to the -CHOCOCH₃ proton of the vinyl acetate monomer unit at $\delta = 4.8$ ppm as reported elsewhere previously [15, 16].

ACKNOWLEDGMENTS

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References

1. Georges MK, Veregin RPN, Kazmaier PM, Hamer GK (1993) Narrow molecular weight resins by a free-radical polymerization process. *Macromolecules* 26:2987–2988. <https://doi.org/10.1021/ma00063a054>
2. Hawker CJ, Bosman AW, Harth E (2001) New polymer synthesis by nitroxide mediated living radical polymerizations. *Chem Rev* 101:3661–3688. <https://doi.org/10.1021/cr990119u>
3. Sciannamea V, Jérôme R, Detrembleur C (2008) In-situ nitroxide-mediated radical polymerization (NMP) processes: their understanding and optimization. *Chem Rev* 108: 1104–1126. <https://doi.org/10.1021/cr0680540>
4. Matyjaszewski K, Xia J (2010) Atom transfer radical polymerization. *Prog Chem* 22:2079–2088. <https://doi.org/10.1002/9783527809080.cataz01278>
5. Perrier S, Takolpuckdee P (2005) Macromolecular design via reversible addition-fragmentation chain transfer (RAFT)/ xanthates (MADIX) polymerization. *J Polym Sci Part A Polym Chem* 43:5347–5393. <https://doi.org/10.1002/pola.20986>
6. Debuigne A, Poli R, Jérôme C et al (2009) Overview of cobalt-mediated radical polymerization: roots, state of the art and future prospects. *Prog Polym Sci* 34:211–239. <https://doi.org/10.1016/j.progpolymsci.2008.11.003>
7. Demarteau J, Debuigne A, Detrembleur C (2019) Organocobalt complexes as sources of carbon-centered radicals for organic and polymer chemistries. *Chem Rev* 119:6906–6955. <https://doi.org/10.1021/acs.chemrev.8b00715>
8. Sproncken CCM, Surís-Valls R, Cingil HE et al (2018) Complex coacervate core micelles containing poly(vinyl alcohol) inhibit ice recrystallization. *Macromol Rapid Commun.* <https://doi.org/10.1002/marc.201700814>
9. Congdon TR, Notman R, Gibson MI (2017) Synthesis of star-branched poly(vinyl alcohol) and ice recrystallization inhibition activity. *Eur Polym J* 88:320–327. <https://doi.org/10.1016/j.eurpolymj.2017.01.039>
10. Vail NS, Stubbs C, Biggs CI, Gibson MI (2017) Ultralow dispersity poly(vinyl alcohol) reveals significant dispersity effects on ice recrystallization inhibition activity. *ACS Macro Lett* 1001–1004. <https://doi.org/10.1021/acsmacrolett.7b00595>
11. Debuigne A, Warnant J, Jérôme R et al (2008) Synthesis of novel well-defined poly(vinyl acetate)-b-poly(acrylonitrile) and derivatized water-soluble poly(vinyl alcohol)-b-poly(acrylic acid) block copolymers by cobalt-mediated radical polymerization. *Macromolecules* 41. <https://doi.org/10.1021/ma702341v>
12. Wang Z, Vertruyen B, Taghipour H et al (2021) CO₂-derived methylene oxazolidinone: a platform building block for functionalizing ethylene-vinyl alcohol copolymers. *Macromolecules* 54:10415–10427. <https://doi.org/10.1021/acs.macromol.1c01895>
13. Wang Z, Poli R, Detrembleur C, Debuigne A (2019) Organometallic-mediated radical (Co)-polymerization of
14. γ -methylene- γ -butyrolactone: access to
15. pH-responsive poly(vinyl alcohol) derivatives. *Macromolecules* 52:8976–8988. <https://doi.org/10.1021/acs.macromol.9b01838>

16. Scholten PBV, Demartean J, Gennen S et al (2018) Merging CO₂-based building blocks with cobalt-mediated radical polymerization for the synthesis of functional poly(vinyl alcohol)s. *Macromolecules* 51:3379–3393.
17. <https://doi.org/10.1021/acs.macromol.8b00492>
18. Debuigne A, Caille J-R, Detrembleur C, Jérôme R (2005) Effective cobalt mediation of the radical polymerization of vinyl acetate in suspension. *Angew Chemie Int Ed* 44:3439–3442. <https://doi.org/10.1002/anie.200500112>
19. Debuigne A, Caille JR, Jerome R (2005) Highly efficient cobalt-mediated radical polymerization of vinyl acetate. *Angew Chemie Int Ed* 44:1101–1104. <https://doi.org/10.1002/anie.200461333>