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# Water-assisted and protein-initiated fast and controlled ring-opening polymerization of proline *N*-carboxyanhydride

Yali Hu[1,2], Zi-You Tian[1], Wei Xiong[1], Dedao Wang[3], Ruichi Zhao[1], Yan Xie[3], Yu-Qin Song[3], Jun Zhu[3] and Hua Lu[1,*]

[1]Beijing National Laboratory for Molecular Sciences, Center for Soft Matter Science and Engineering, Key Laboratory of Polymer Chemistry and Physics of the Ministry of Education, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China; [2]Peking-Tsinghua Center for Life Sciences, Academy for Advanced Interdisciplinary Studies, Peking University, Beijing 100871, China and [3]Key Laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Lymphoma, Peking University Cancer Hospital and Institute, Beijing 100142, China

*Corresponding author. E-mail: chemhualu@pku.edu.cn

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## ABSTRACT

The production of polypeptides via the ring-opening polymerization (ROP) of *N*-carboxyanhydride (NCA) is usually conducted under stringent anhydrous conditions. The ROP of proline NCA (ProNCA) for the synthesis of poly-L-proline (PLP) is particularly challenging due to the premature product precipitation as polyproline type I helices, leading to slow reactions for up to one week, poor control of the molar mass and laborious workup. Here, we report the unexpected water-assisted controlled ROP of ProNCA, which affords well-defined PLP as polyproline II helices in 2–5 minutes and almost-quantitative yields. Experimental and theoretical studies together suggest the as-yet-unreported role of water in facilitating proton shift, which significantly lowers the energy barrier of the chain propagation. The scope of initiators can be expanded from hydrophobic amines to encompass hydrophilic amines and thiol-bearing nucleophiles, including complex biomacromolecules such as proteins. Protein-mediated ROP of ProNCA conveniently affords various protein-PLP conjugates via a grafting-from approach. PLP modification not only preserves the biological activities of the native proteins, but also enhances their resistance to extreme conditions. Moreover, PLP modification extends the elimination half-life of asparaginase (ASNase) 18-fold and mitigates the immunogenicity of wt ASNase >250-fold (ASNase is a first-line anticancer drug for lymphoma treatment). This work provides a simple solution to a long-standing problem in PLP synthesis, and offers valuable guidance for the development of water-resistant ROP of other proline-like NCAs. The facile access to PLP can greatly boost the application potential of PLP-based functional materials for engineering industry enzymes and therapeutic proteins.

**Keywords:** proline, PPII helix, ring-opening polymerization, *N*-carboxyanhydride, protein-polymer conjugates

## INTRODUCTION

Proline (Pro) is the only proteinogenic amino acid bearing a secondary amine, which results from a circular side chain that loops back and reconnects with the backbone nitrogen. This pyrrolidine ring creates a sterically hindered nitrogen and constrains the conformation of both the Pro and its preceding amino acid residue. Not surprisingly, the Pro-Pro junction is even more restricted; as an extreme case, poly-L-proline (PLP) is a well-known rigid 'molecular ruler' that exists either as all-*cis* right-handed type I (PPI) helices in common organic solvents or as all-*trans* left-handed type II (PPII) helices in aqueous solution [1,2]. In naturally occurring proteins, proline-rich regions (PRRs) in PPII helices play important roles in regulating protein–protein and protein–nucleic acid interactions, signaling, mechanical elasticity, transcriptional activation, immune response, etc. [3–6]. In materials science, there is also a rapidly growing interest in proline- or hydroxyproline-derived polymers [7–9]. PLP derivatives have also demonstrated utility as mimics of antifreeze protein [10], building blocks for hierarchical self-assembly [11], templates for controlling

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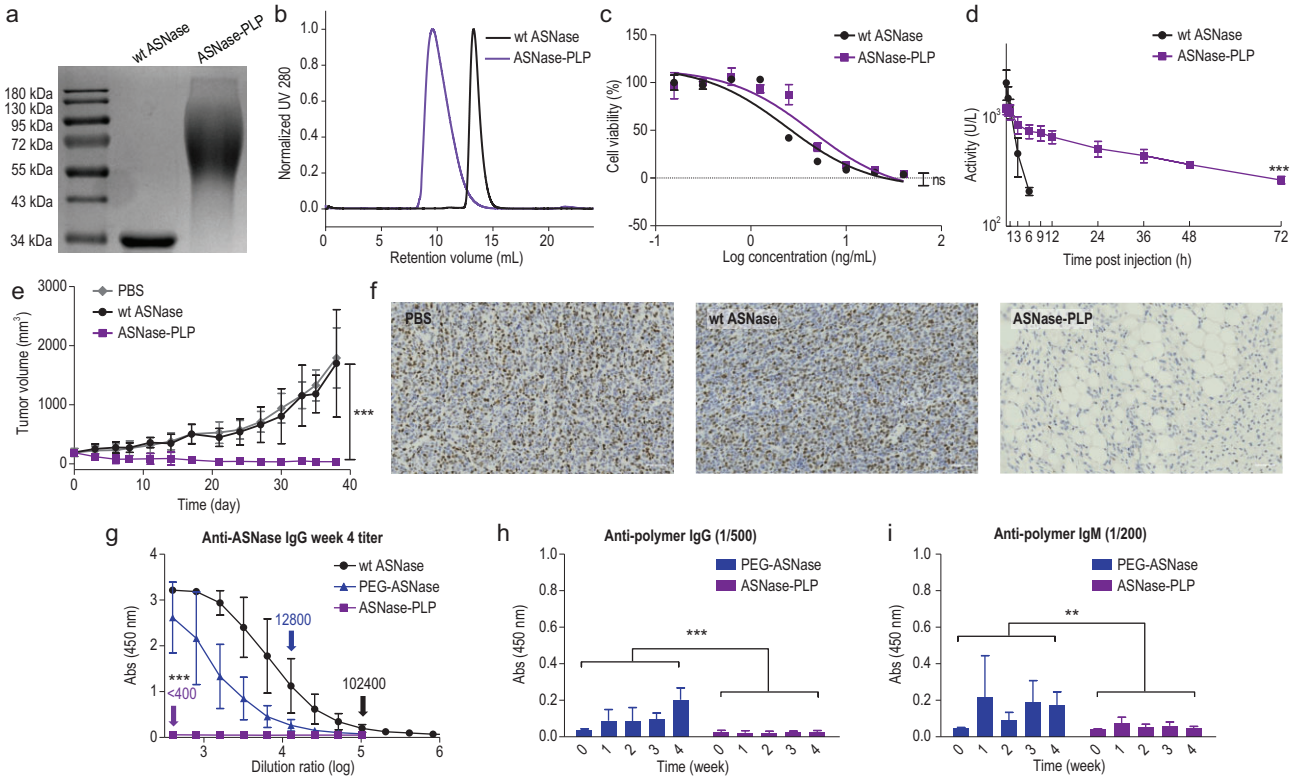


**Figure 6.** Pharmacological properties and immunogenicity of the ASNase-PLP conjugate. (a) SDS-PAGE and (b) aqueous SEC characterization of ASNase-PLP prepared by ASNase-mediated ROP of ProNCA. (c) *In vitro* cytotoxicity of ASNase-PLP on a human NK/T lymphoma cell line NKYS; $n = 3$. (d) Pharmacokinetic profile of intravenously infused ASNase-PLP in SD rats; $n = 4$; the enzymatic activity, obtained using Nessler's reagent (Merck, Germany), was employed to measure the plasma concentration of ASNase. (e) NKYS tumor growth curves and (f) immunohistochemistry (anti-human ki67) of tumor sections after receiving biweekly intraperitoneal treatments of PBS, wt ASNase (15 U/mouse) or ASNase-PLP (15 U/mouse). Scale bars, 50 $\mu$m. Six-week-old B-NDG mice were subcutaneously inoculated with NKYS cells ($6.0 \times 10^6$) and randomized ($n = 8$ for each group) when the tumors reached ~300 mm$^3$ (day 0). (g) ELISA analysis of the titers of anti-ASNase IgG in the week-4 antisera drawn from wt ASNase-, ASNase-PEG- or ASNase-PLP-infused SD rats. (h, i) ELISA analysis of the changes of anti-polymer (h) IgG and (i) IgM levels in the antisera drawn from wt ASNase-, ASNase-PEG- or ASNase-PLP-infused SD rats. For the immunization, SD rats ($n = 4$ for each group) were subcutaneously infused with wt ASNase, ASNase-PEG or ASNase-PLP (200 U/kg) once every week for totally four weeks. Antisera were drawn every week before each injection. For the ELISA assay, the plates were coated with (g) wt ASNase, a PLP-interferon conjugate (to detect anti-PLP antibodies) and (h, i) a PEG-interferon conjugate (to detect anti-PEG antibodies). Data are represented as mean ± SD. *P* values are determined by ANOVA (ns = no significant difference; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

killing (Fig. 6f). All mice exhibited remarkable tolerance of the drug conjugate at the applied dose based on the tracking of body weight (Fig. S24) and histological assessment of major organs (Fig. S25). These results vividly showed that extending the blood half-life of ASNase could significantly improve its anti-tumor efficacy *in vivo*. ASNase is a bacterial enzyme with extremely high immunogenicity, and the related side effects cannot be completely eliminated in clinical practice even through PEG modification. More worrisome is the hapten nature of PEG itself, which induces the production of anti-PEG antibodies after coupling with ASNase, which in turn leads to hypersensitivity and an accelerated blood clearance (ABC) effect. To interrogate whether PLP conjugation was a better alternative, we injected the rats with ASNase-PLP or ASNase-PEG subcutaneously once a week and collected antisera each time before the injection. Enzyme linked immunosorbent assay (ELISA) analysis revealed that the titer of anti-ASNase IgG in the ASNase-PLP antisera harvested at week four was <400, compared to 12 800 and 102 400 for the antisera of ASNase-PEG and wt ASNase, respectively (Fig. 6g). Moreover, the levels of anti-polymer IgM and IgG in the ASNase-PLP antiserum were also significantly lower than in ASNase-PEG antiserum (Fig. 6h and i).

## DISCUSSION AND CONCLUSIONS

PPII helices are among the most prevalent structural and functional motifs in proteins. Synthetic PPII-helical PLP can emulate the unique structural and functional roles of PPII helices, ultimately leading to the creation of novel biochemical materials.



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However, conventional methods for PLP synthesis are slow and inefficient, require stringent moisture-free conditions, allow very little control of $M_n$ and $Đ$, and often result in premature product precipitation as PPI helices. In the current study, we demonstrated that controlled and dramatically accelerated ROP of ProNCA could be easily achieved by simply adding water as a co-solvent (Fig. 1). Notably, to obtain accurate $M_n$ and $Đ$ information of the PLP, we employed an aqueous SEC system equipped with both multi-angle light scattering (MALS) and refractive index detectors, which allowed us to acquire the d$n$/d$c$ value of the PLP and calculate the absolute $M_n$. One notable limitation of many previous methods is that the scope of initiators was restricted to hydrophobic amines. This limitation was easily overcome in this study for the obvious reason that water is a good solvent for many small molecules and biomacromolecules. Indeed, we successfully expanded the scope of initiators to hydrophilic amine- and thiol-bearing nucleophiles, including complex biomacromolecules such as proteins (Figs 4–6).

Moisture-tolerant ROP of NCAs has gained considerable attention in recent years with many notable advances. The unique feature of our work is that water played multiple beneficial roles in our reaction system rather than being a detrimental factor. Key contributing factors to the excellent reaction control that we observed included enhanced solubility of PLP in water and the unexpected water-assisted acceleration of the ROP, which allowed it to kinetically outcompete the monomer hydrolysis. DFT results revealed the crucial mechanistic role of water molecules in facilitating a hydrogen shift that lowered the energy barrier of the chain propagation by a substantial ∼7.1 kcal/mol (Fig. 3). However, it should be noted that at $[\text{ProNCA}]_0/[\text{I}]$ ratios higher than 200/1, the hydrolysis of ProNCA became significant.

Synthetic polypeptides have been used for protein conjugation, i.e. protein PEPylation, for improved pharmacological properties [43,51,54,57–61]. But the function of PLP for protein modification is yet to be fully exploited. Here, we showed that PLP was effective in protecting the conjugated protein from denaturation under harsh conditions such as high temperature and exposure to organic solvents. Interestingly, the PLP-grafted EGFP fully preserved the EGFP fluorescence and DHFR catalytic activity. There are three plausible reasons for this result. First, the fast kinetics of ROP allowed product generation within 10 min, and thus minimized the detrimental effects posed by exposure to the organic solvent. Second, it appeared that the growth of PLP on the surface of DHFR enhanced the resistance to organic solvent dramatically (Fig. 5d). Third, the substrates of the DHFR-catalyzed reaction were small molecules, whose diffusion and binding to the catalytic pocket was generally insensitive to the steric hindrance of PLP. Although this grafting-from approach might not be suitable for proteins whose biological functions are dependent on lysine(s) in the active pocket, it could still find broad application in a variety of proteins whose functions are lysine independent. For instance, our results showed that PLP modification substantially improved the pharmacokinetic properties of ASNase while largely preserving its catalytic activity, which eventually led to enhanced *in vivo* antitumor efficacy. Most interestingly, our results unambiguously proved that PLP could not only reduce the immunogenicity of the protein covalently attached to it, but its antigenicity *per se* was also lower than PEG. These results indicate that PLP, which is neutral in charge, biodegradable and completely natural, holds great potential as an outstanding alternative to the non-degradable PEG, whose clinical limitations have been increasingly recognized in recent years [54,60,62–65].

Overall, this work provides a simple means of solving a longstanding problem in PLP synthesis and offers valuable guidance for the development of water-resistant ROP of other NCAs. The availability of PLP could facilitate our understanding of the biophysical and biological roles of PRRs in natural proteins and greatly boost the application potential of PLP-based functional materials for engineering of industrial enzymes and therapeutic proteins.

## SUPPLEMENTARY DATA

Supplementary data are available at *NSR* online.

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## AUTHOR CONTRIBUTIONS

Y.L.H. and H.L. conceived the idea, designed the experiments, analyzed the data and wrote the manuscript. Y.L.H. conducted most experiments; Z.Y.T. performed the DFT calculation; W.X. performed all of the MALDI-TOF analyses; D.D.W. performed the antitumor efficacy study; R.C.Z. performed ELISA; Y.X., Y.Q.S. and J.Z. provided resources for the ASNase studies. All authors read and approved the final version of the manuscript.

***Conflict of interest statement.*** None declared.

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