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Controlled Synthesis and Enzyme-Induced Hydrogelation of Poly(L-phosphotyrosine)s via Ring-Opening Polymerization of α-Amino Acid *N*-Carboxyanhydride

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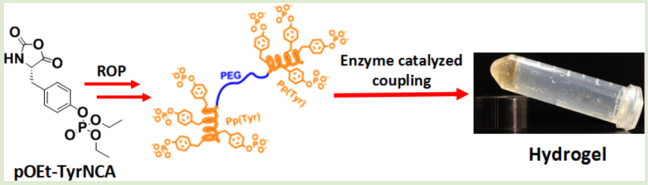
Supporting Information

ABSTRACT: Tyrosine phosphorylation is an important post-translational modification (PTM) that governs numerous cellular processes. Constructing synthetic phosphotyrosine polypeptides helps expand the horizon of our understanding regarding the fundamental aspects and biological consequences of this PTM. Here, we report the synthesis of a novel monomer, *O*-diethylphospho L-tyrosine *N*-carboxyanhydride (pOEt TyrNCA), whose ring-opening polymerization (ROP) mediated by hexamethyldisilazane (HMDS) leads to poly(L-phosphotyrosine) (P(pTyr)) derivatives with controllable molecular weights and narrow molecular-weight distributions. Moreover, $P(pTyr)_{15}$-*b*-PEG-*b*-$P(pTyr)_{15}$ triblock copolymer (TBP15) undergo sol−gel transition in the presence of alkaline phosphatase. The stiffness of the gel is reinforced by the addition of horseradish peroxidase and hydrogen peroxide. These phosphotyrosine-mimicking polymers are realized for the first time by NCA polymerization and are promising materials for a variety of biological applications.



Synthetic polypeptides that mimic structural motifs of natural-occurred proteins are highly attractive for their biodegradability, excellent biocompatibility and tunable properties. This bioinspired approach has led to numerous synthetic polypeptides[1] prepared by the ring-opening polymerization (ROP) of α-amino acid *N*-carboxyanhydrides (NCAs).[2] Coincident with the emergence of epigenetics, polypeptides composed of protein post-translational modification (PTM) structures have drawn growing interest in the past decade. As such, complex glycopolypeptides mimicking protein glycosylation, one of the most important PTMs, have been realized, and their biological functions and self-assembly have been extensively studied.[3]

Phosphorylation of protein is another important PTM that commonly occurs at serine, threonine, and tyrosine residues. Among which, protein tyrosine phosphorylation is particularly intriguing due to its pivotal role in numerous signaling cascades that govern virtually all cellular processes including growth, apoptosis, and differentiation. Often, an aberrant level of tyrosine phosphorylation or dephosphorylation is a hallmark of diseases such as cancer and autoimmune disorders, making regulation of this process one of the most prevalent approaches for drug discovery.[4] Because of the biological importance of phosphorylation, several attempts have been made to generate analogues of poly(L-phosphoserine) and poly(L-phosphothreonine) prepared via the ROP of novel NCAs bearing phosphate or phosphonate side chains.[5] The application of these poly(L-phosphoserine) in calcium biomineralization[6] have also been reported in the past decade. To our surprise, poly(L-phosphotyrosine)s (P(pTyr)s) have not been accomplished by NCA polymerization.

We are enthused about P(pTyr)s for a number of reasons. First, we envision that P(pTyr)s are arguably among the most negatively charged polymers with excellent solubility in aqueous buffers across a broad pH range based on the pendant phosphate groups. Moreover, we reason that enzymatic dephosphorylation of P(pTyr)s is worth to explore and achievable by protein tyrosine phosphatases such as alkaline phosphatase (ALP), a sequence-independent ectoenzyme that overexpressed in certain type of tumor cells. To this end, small molecules containing phosphotyrosine have been engineered as intelligent ALP-sensitive gelators for the delivery of many therapeutic agents such as nonsteroidal anti-inflammatory drug (NSAID)[7] and taxol,[8] as well as anti-HIV DNA vaccines.[9] The ALP triggered in situ dephosphorylation has also been exploited very recently for the selective induction of apoptosis of cancer cells by forming pericellular gels.[10] Since only small molecular gelators have been reported to date, we believe the exploration on P(pTyr) as a macromolecular gelator will be a good addition.

Herein, we report the synthesis of *O*-diethylphospho L-tyrosine *N*-carboxyanhydride (pOEt-TyrNCA) and its ROP to produce biomimetic P(pTyr)s. The P(pTyr)s are highly soluble in water and nontoxic to human cell lines below 1000 μg/mL.

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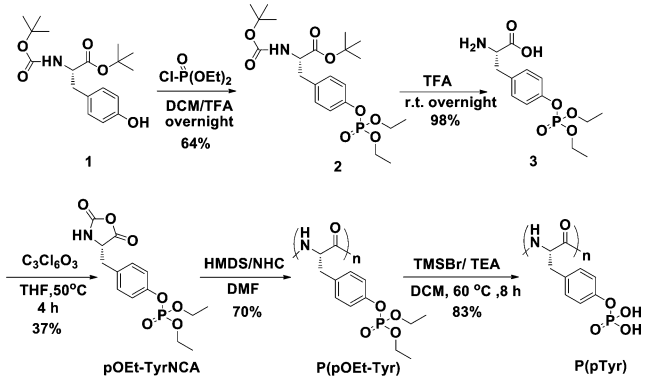
Moreover, we show that the P(pTyr)-*b*-PEG-*b*-P(pTyr) triblock copolymer forms a hydrogel in the presence of ALP, a result of the enzymatic dephosphorylation of P(pTyr) that induces physical aggregation of the insoluble poly(L-tyrosine) (PLT). We further show in this work that the storage modulus of the hydrogel can be significantly enhanced by horseradish peroxidase (HRP) and hydrogen peroxide that covalently cross-link phenol rings.[11]

Due to the synthetic challenge of phosphoserine NCA and the solubility problem of its resulting polymers, phosphonate analogues that are easier to handle and modify have been used as alternative choices,[5b] which do not reflect wild-type phosphoserine with full fidelity. We designed pOEt-TyrNCA because it eventually leads to a polymer structure exactly identical to phosphotyrosine that appeared in proteins after its polymerization and subsequent removal of ethyl groups. As shown in Scheme 1, pOEt-TyrNCA was readily synthesized

Scheme 1. Synthesis of pOEt-TyrNCA and P(pTyr)s



within three steps. Briefly, commercially available Boc-Tyr-O*t*Bu (**1**) was reacted with diethyl chlorophosphate to generate Boc-Tyr(PO_3Et_2)-O*t*Bu (**2**), which underwent overnight deprotection in trifluoroacetic acid (TFA) at room temperature to afford the corresponding *O*-diethylphospho L-tyrosine (**3**). Crude pOEt-TyrNCA monomer was obtained by cyclization of **3** in dry THF using triphosgene, a standard procedure generating NCAs. Notably, pOEt-TyrNCA was purified by a regular flash silica column in an ambient atmosphere, followed by recrystallization once in THF−hexane in the glovebox. The monomer was fully characterized by ^{1}H and ^{13}C NMR (Figure S1), mass spectrometry (Figure S2), and FT-IR spectrometry (Figure S3).

Next, we studied the ROP of pOEt-TyrNCA in a glovebox. Initial attempts to polymerize pOEt-TyrNCA using hexamethyldisilazane (HMDS)[12] led to slow reaction, likely due to the steric hindrance of the side chain phenyl ring. To boost the polymerization, a catalytic amount of 1,3-dimesitylimidazol-2-ylidene (NHC, NHC/HMDS = 0.3/1) that works as an acyl transfer/activation reagent and has been routinely used in the ROP of lactide, lactone, and also *N*-substituted glycine NCAs,[13] was added to the reaction solution at room temperature. To our delight, the polymerizations all reached over 95% NCA conversion with the aid of NHC (determined by FT-IR, Figure S4). Upon elevating the monomer/initiator (M/I = NCA/HMDS) ratios from 50/1 to 200/1, we obtained poly(*O*-diethylphospho L-tyrosine)s (P(pOEt-Tyr)s) with linearly increased molecular weights (MWs, M_n) and molecular-weight distributions (MWDs = M_w/M_n) ranging from 1.16 to 1.34, as measured by gel permeation chromatography (GPC; Figure 1). Remarkably, the obtained MWs of P(pOEt-Tyr)s were all less

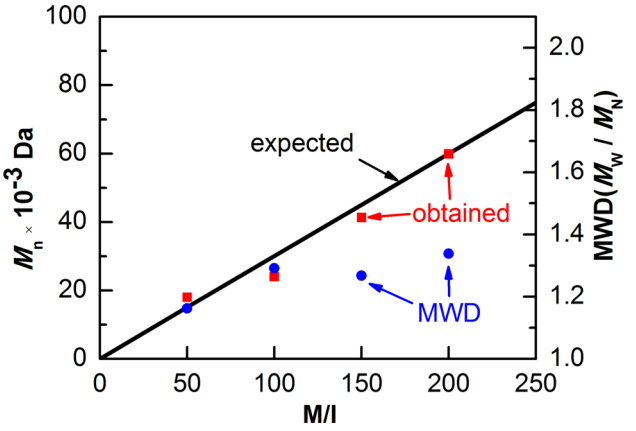


Figure 1. Plot of obtained MWs (red squares) and MWDs (blue circles) of P(pOEt-Tyr)s as a function of M/I (NCA/HMDS) ratios. The black curve represents the expected MWs of P(pOEt-Tyr)s at different M/I ratios.

than 20% deviation from the expected MWs. Particularly, the obtained MW of the P(pOEt-Tyr) at the M/I ratio of 200/1 was 59.9 × 10^3 Da, exactly the number we expected.

P(pOEt-Tyr) (^{1}H NMR spectrum in Figure S5A) showed remarkable solubility in common organic solvents such as dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), and tetrahydrofuran (THF), a notable advantage compared with the insoluble poly(*O*-diethyl L-phosposerine). P(pOEt-Tyr) adopted helical conformation in THF solution as determined by FT-IR analysis (Figure S4). Deprotection of P(pOEt-Tyr) with trimethylsilyl bromide (TMSBr) in DCM afforded crude P(pTyr), which was continually purified by extensive dialysis. The completion of the deprotection was confirmed by the disappearance of the ethyl peaks, as shown in the ^{1}H NMR spectrum of P(pTyr) (Figure S5B). No detectible dephosphorylation occurred during the deprotection, as only one set of phenyl hydrogens was observed in the ^{1}H NMR spectrum of P(pTyr). As expected, P(pTyr)s are highly soluble in various aqueous buffers, with pH ranging from 2 to 10. The circular dichroism (CD) spectra of P(pTyr) (Figure S6), which displayed different patterns from typical polypeptides due to the overlapping of optical rotation of the side chain tyrosyl and the backbone amide chromophores,[14] exhibited a conformational change as the pH was slowly titrated to 1.0. This is attributed to likely a disordered−ordered transition responding to the protonation of the side chain phosphate that tunes the polymer from ionic to neutral state. FT-IR analysis of the mulls of P(pTyr) lyophilized from pH 1.0 (slow titration) showed a characteristic amide I peak at 1633 cm^{-1} (Figure S7), implying the polymer adopts β-sheet conformation.

To evaluate the cytotoxicity of P(pTyr), HeLa (a human cervical cancer cell line) and HUVEC (an immortalized human endothelium cell line) were incubated for 48 h with various concentrations of $P(pTyr)_{200}$, a 200-mer of P(pTyr). The relative viabilities of $P(pTyr)_{200}$-treated cells were measured by MTT assay and normalized to viability of medium-treated cells. Figure 2 depicts that $P(pTyr)_{200}$ showed no significant toxicity to both HeLa and HUVEC cells at concentrations up to 200 μg/mL, whereas marginal toxicities (20−30%) were observed at 1000 μg/mL, the highest concentration we tested. This study

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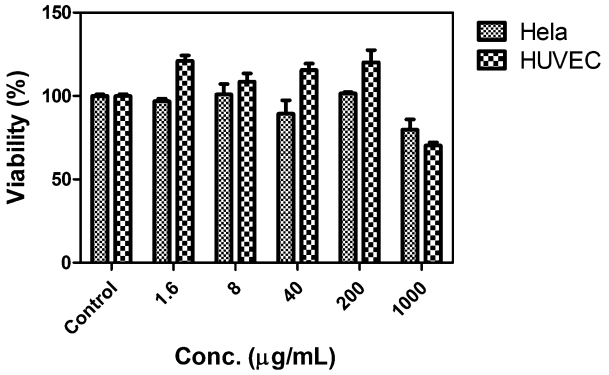
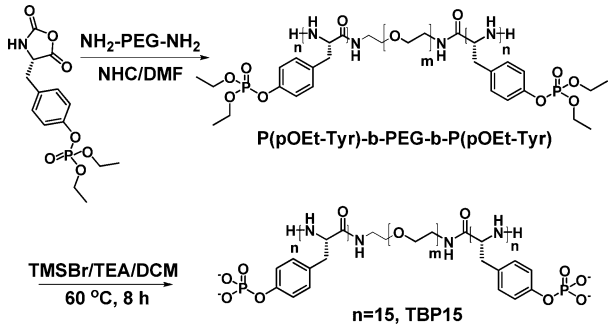


Figure 2. Viabilities of cells treated with $P(pTyr)_{200}$. HeLa and HUVEC cells were incubated with $P(pTyr)_{200}$ at denoted concentrations for 48 h before the cell viabilities were measured by MTT assay. Viabilities were normalized to control group that incubated with medium only, and data were shown as means ± SD (standard deviation); $n = 3$.

implied that P(pTyr) is nontoxic and relatively safe at appropriated concentrations for biological applications.

To obtain polymeric ALP-sensitive gelators, a triblock polymer denoted as TBP15, in which a polyethylene glycol (PEG, MW 5000 Da) was flanked by two 15-mer of P(pTyr) domains, was synthesized as shown in Scheme 2. Briefly,

Scheme 2. Synthesis of Triblock Polymer TBP15



bisamine-functionalized PEG (NH_2-PEG-NH_2) was utilized as a macroinitiator to polymerize pOEt-TyrNCA at NCA/NH_2 molar ratio of 15/1 in the presence of catalytic NHC and yields triblock polymer $P(pOEt\text{-}Tyr)_{15}$-*b*-PEG-*b*-$P(pOEt\text{-}Tyr)_{15}$. Deprotection of $P(pOEt\text{-}Tyr)_{15}$-*b*-PEG-*b*-$P(pOEt\text{-}Tyr)_{15}$ generated water-soluble $P(pTyr)_{15}$-*b*-PEG-*b*-$P(pTyr)_{15}$, denoted as TBP15, in high yield. The MW of the polymer was determined by both GPC and ^{1}H NMR (Figures S8 and S9).

To generate hydrogel by ALP-catalyzed dephosphorylation (denoted as TBP15-ALP hydrogel), TBP15 (8 wt %) in Tris·HCl buffer (pH ∼ 8.0) was incubated with ALP at 37 °C. The viscosity of the mixture increased gradually, and a soft gel formed after overnight incubation, likely due to the dephosphorylation of P(pTyr)s that induces aggregation of the resulting PLT domain (Figure 3A). To confirm the hypothesis, TBP15-ALP hydrogel was dialyzed against ultrapure water, lyophilized, and analyzed by ^{1}H NMR and FT-IR. A total of ∼85% of the P(pTyr) segment was dephosphorylated according to the ^{1}H NMR spectrum (Figure S10) of the remaining powder redissolved in alkaline D_2O. The solid phase FT-IR spectrum (Figure S11) of the same powder showed an amide I peak at 1639 cm^{-1}, suggesting a β-sheet conformation

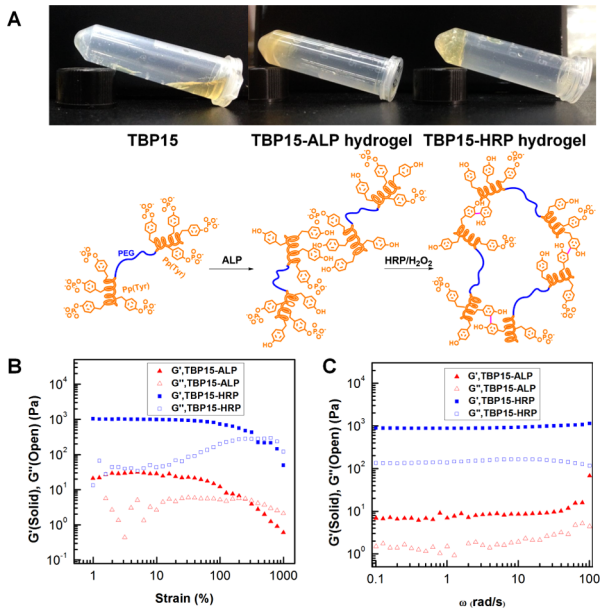


Figure 3. Photographs and cartoon illustration of TBP15 solution, TBP15-ALP (8 wt %), and TBP15-HRP (4 wt %) hydrogels (A). Strain sweep with a frequency of 6 rad/s (B) and frequency sweep with a strain of 40% (C) of TBP15-ALP and TBP15-HRP hydrogels. G' of TBP15-ALP (red triangles); G'' of TBP15-ALP (red open triangles); G' of TBP15-HRP (blue squares); G'' of TBP15-HRP (blue open squares).

that was similar to the previously reported PLT.[15] To improve the stiffness of the gel and the gelation kinetics, we sought to chemically cross-link the 2′ positions of two dephosphorylated phenol rings by HRP enzymatic catalysis (Figure 3A). As such, ALP, HRP, and hydrogen peroxide were mixed simultaneously with TBP15 (4 wt %) under similar conditions as described for the TBP15-ALP hydrogel. Remarkably, a robust gel (denoted as TBP15-HRP) formed within 1.5 h of incubation with the aid of HRP, which was considerably faster than the hydrogel generation with only ALP. To examine the mechanical properties of the hydrogels, oscillatory rheology experiments were performed on both TBP15-ALP and TBP15-HRP hydrogels (Figure 3B,C). The dynamic strain sweep study (Figure 3B) with a ω of 6.0 rad·s^{-1} revealed that TBP15-ALP and TBP15-HRP hydrogels have a critical strain[8] of ∼50 and ∼80%, respectively (sweep range: 1−1000%). A subsequent frequency sweep study (Figure 3C) with a strain of 40% showed that both TBP15-ALP and TBP15-HRP hydrogels have significantly higher storage moduli (G') than their own loss moduli (G'') and very weak frequency dependence of G's, reflecting the gel state. Notably, the G' of the TBP15-HRP hydrogel (∼882 Pa at 2 rad/s) is almost 2 orders of magnitude higher than the G' of TBP15-ALP hydrogel (∼7.2 Pa at 2 rad/s), as shown in the frequency sweep study (Figure 3C), necessitating the HRP-catalyzed cross-linking in order to gain higher moduli hydrogels.

In conclusion, we demonstrated in this communication that P(pTyr), mimicking naturally occurring phosphotyrosine, was realized for this first time by the ROP of pOEt-TyrNCA. P(pTyr)s showed excellent solubility in water and were not toxic to cells at concentrations below 1000 μg/mL. ALP and HRP cascade enzyme reactions generated a robust hydrogel of block copolymers TBP15. We are currently investigating the

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applications of P(pTyr)s for a number of biological and biomedical purposes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmacrolett.5b00429.

Materials and methods, instrumentation, and ^{1}H and ^{13}C NMR, FT-IR, ESI-MS, and GPC data (PDF)

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Notes

The authors declare no competing financial interest.

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