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ARTICLE TYPE

Environmentally Responsive Amino Acid-Bioconjugated Dynamic Covalent Copolymer as a Versatile Scaffold for Conjugation

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Abstract: Biodynamers display the properties of biomolecules with the adaptive nature of dynamic covalent polymers and have great potential as "smart" materials. The bioconjugation of L-tyrosine hydrazide to the end of bisaldehyde-end-functionalized thermoresponsive copolymer chains via reversible acylhydrazone linkage generates a biodynamer with thermo/pH- responsive and adaptive features. Because of the introduction of amino acid end group, the biodynamer has an isoelectric point (IEP) at pH 4.70 and possesses a pH-dependent LCST. The property of the biodynamer is tunable through chain exchange with other hydrazine or hydrazide molecule. The biodynamer endowed biological recognition by exchange with biotin hydrazide. The complex formed by biotinylated dynamer and streptavidin demonstrates pH-responsive feature. Because of the versatile reactivity of phenolic moiety, this biodynamer provides a reactive scaffold for further modification. The cyclic diazodicarboxamide is attached to the biodynamer through the tyrosine-click reaction. Protein bioconjugates with pH-responsive and adaptive features are prepared via HRP-catalyzed coupling reaction.

Introduction

The implementation of dynamic covalent chemistry (DCC) in polymer science generates "smart" dynamers possessing environmentally responsive and adaptive characteristics. Various dynamers with thermoresponsive, chemoresponsive, photoresponsive or electroresponsive properties and self/re-healing gels were constructed on the basis of reversible covalent bonds.[1-3] Lehn and coworkers incorporated biologically relevant substances into dynamers and developed biodynamers which combined the functional properties of biomolecuels with the adaptive nature of dynamic polymers. For example, glycodynamers, dynamic analogues of nucleic acids and dynamic analogues of proteins have been developed in Lehn's group.[4-7] These biodynamers can be considered as the dynamic analogues of natural macromolecules. The reversible nature of dynamic covalent bond enables the biodynamers to undergo marked changes in their physical and chemical properties by exchanging and reshuffling their building blocks in response to external stimuli such as temperature or pH. The development of dynamic analogues of diverse biomacromolecules may lead to the construction of smart biohybrid materials that have numerous applications in the areas of advanced materials, biology and medicine.

Tyrosine is one of the most versatile amino acids because of the physicochemical character and the chemical reactivity of its side chain phenolic moiety. Since tyrosine side chain can be involved in hydrogen bonding, π-π interactions and cation-π interactions, tyrosine residues are widely used in the molecular recognition site of antibodies.[8] Tyrosine residues also provide attractive targets for protein modifications, as these hydrophobic residues are present with intermediate frequency on protein surfaces, and the reactivity of the phenolic group is orthogonal to that of cysteine, lysine, and carboxylate-containing residues.[9] Tyrosine residues have unique reactivity because of the acidic proton of the phenol ring.[10] The alkyation or acylation reaction of tyrosine under basic conditions proceeds at the oxygen. Under acidic conditions, an ene-like reaction occurs at a carbon atom on the aromatic ring. There have been several reports on tyrosine-mediated protein modifications using a chemical oxidative reaction,[11-13] a Mannich-type reaction,[9,14,15] a diazonium coupling reaction[16-19] and a rapid tyrosine-click reaction.[20-22] Intrigued by the possibility of using ideas from the protein modification through tyrosine, we sought to introduce tyrosine moiety into the polymer to create a facile bioconjugation strategy for the synthetic polymers through tyrosine. Protein-polymer bioconjugates are hybrid materials that integrate the properties and functions of biomacromolecules and synthetic polymers. These biohybrid materials are promising building blocks for the preparation of functional nanometer-sized materials,[23,24] which have numerous potential applications in biotherapeutics, biotechnology, and nanotechnology.[25,26]

In the previous studies, we constructed thermoresponsive dynamer, anionic dynamer and the multi-functionalizable core-cross-linked nanoparticles on the basis of reversible acylhydrazone bond.[27-29] Here, we presented a thermo/pH-responsive tyrosine-bioconjugated dynamic covalent copolymer via acylhydrazone linkage. L-Tyrosine hydrazide was attached to bisaldehyde-functionalized copolymer via reversible acylhydrazone linkage to obtain the biodynamer. This biodynamer demonstrates adaptive nature of dynamic system

combined with the function of amino acid moiety. Because of the versatile reactivity of phenolic moiety, this biodynamer can be further modified by tyrosine-click reaction and HRP-mediated coupling reaction. The coupling of BSA and the biodynamer generated protein bioconjugate with pH-responsive and adapative features.

Experimental Section

Materials.

Di(ethylene glycol) ethyl ether acrylate (DEGA, Sigma, 98%) and poly(ethylene glycol) methyl ether acrylate (PEGA, M_n 480 g/mol, Sigma) were purified by passing through a basic alumina column. L-Tyrosine hydrazide (Sigma, 98%), 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD, Sigma, 97%), 2-hydrazinopyridine (Alfa, 98%), bovine serum albumin (BSA) and horseradish peroxidase (HRP) were used as received. Azobis(isobutyronitrile) (AIBN) was recrystallized twice from methanol. Bisaldehyde-modified chain transfer agent CTA-bisCHO was synthesized according to the method reported in the literature.[27]

Characterization.

^{1}H NMR measurements were recorded on a Varian UNITY- plus 400 M nuclear magnetic resonance spectrometer using $CDCl_3$ or d_6-DMSO as the solvent. The number-average molecular weights (M_n), weight-average molecular weights (M_w), and polydispersities (M_w/M_n) of the polymers were determined by GPC at 35°C with a Waters 1525 chromatograph equipped with a Waters 2414 refractive index detector, where tetrahydrofuran (THF) was used as eluent at a flow rate of 1 mL/min and polystyrene standards were used for calibration. UV−vis spectroscopy was performed on a Shimadzu UV-2450 UV−vis spectrophotometer. Dynamic light scattering (DLS) analysis was conducted on a Zetasizer Nano ZS from Malvern Instruments equipped with a 10 mW HeNe laser at a wavelength of 633 nm at 90° angle. Zeta potential were conducted on a Zetasizer Nano ZS from Malvern Instruments. Transmission electron microscopy (TEM) observations were carried out on a Tecnai G2 F20 electron microscope equipped with a Model 794 CCD camera. The samples were deposited on a carbon-coated copper grid and water was evaporated in air. To increase the contrast, the samples were stained by hydrazine hydrate and OsO_4 vapor.

Synthesis of P(DEGA-co-PEGA) Copolymer C1.

DEGA (0.50 g, 2.7 mmol), PEGA (0.32 g, 0.67 mmol), CTA-bisCHO (85.9 mg, 0.133 mmol), AIBN (2.2 mg, 0.013 mmol) and DMF (2 mL) were added into a flask. The flask was degassed by three freeze-pump-thaw cycles. Then the reaction solution was stirred at 60 °C for 24 h. The reaction was stopped by cooling the solution in ice water and then exposed to air. The P(DEGA-*co*-PEGA) copolymer (C1) was precipitated into n-hexane, centrifuged, and dried overnight under vacuum at room temperature.

Preparation of Tyrosine-Bioconjugated Dynamic Covalent Copolymer D1.

C1 (100 mg, 15 μmol) and L-tyrosine hydrazide (58.5 mg, 0.30 mmol) were dissolved in 5 mL of DMF. Using trifluoroacetic acid (TFA) as the catalyst, the reaction was carried out at 60 °C for 24 h. The excess tyrosine hydrazide was removed by dialysis against $NaHCO_3$ solution (0.01M) for 1 day and distilled water for 2 days (MWCO 1000). The purified tyrosine-bioconjugated dynamic covalent copolymer D1 was recovered by lyophilization.

Modification of D1 through a Tyrosine-Click Reaction.

To a solution of **D1** (10 mg, 1.5 μmol) in 0.1 M pH 8.0 PBS (1.5 mL)/CH_3CN (1.5 mL) was added the 0.1 M solution of PTAD (0.045 mL, 1.5 μmol) in CH_3CN. The resulting solution was stirred at room temperature for 24h. The solution was dialyzed against CH_3CN/PBS (0.02 M pH 8.0, 50 vol%) for 1 day and subsequently against distilled water for 1 days. The modified polymer was recovered by lyophilization.

Synthesis of Biodynamer-Protein Bioconjugate by Horseradish Peroxidase-Catalyzed Tyrosine Coupling Reaction.

D1, BSA and HRP were dissolved in 1 M Tris-HCl (pH 8.0) at final concentrations of 4 mg/mL, 4 mg/mL and 0.05 mg/mL, respectively. The coupling reaction of D1 and BSA was initiated by adding H_2O_2 (30 wt %) solution to the mixture 5 times every 10 min to a final concentration of 50 μM. The reaction mixture was incubated at 37 °C during the addition of H_2O_2 and for a further 24 h following the final addition of H_2O_2. After the incubation, the reaction mixture was purified by dialysis against distilled water for 1 days and ultrafiltration (MWCO 100 kDa).

Chain Exchange Reaction of D1 with 2-Hydrazinopyridine (HP).

D1 (10 mg, 1.4 μmol) and 2-hydrazinopyridine (0.74 mg, 7 μmol) were dissolved in 2 mL of deionized water. Aniline as catalyst was added to the solution. After the pH was adjusted to 5.5, the solution was stirred at 25 °C. The reaction was monitored by the characteristic absorbance of the formed bis-aryl hydrazone at λ 350 nm.[30] After reaction, the solution containing 100 mM aniline was dialyzed against distilled water for 1 day (MWCO 1000), and the product was recovered by lyophilization.

Chain Exchange Reaction of D1 with Biotin Hydrazide.

D1 (20 mg, 3 μmol) was dissolved in acetate buffer (2 mL, 100 mM, pH = 5.5), and the solution of biotin hydrazide (7.7 mg, 3 μmol) in 0.5 mL of DMSO was added. The mixed solution was stirred at 25 °C for 24 h, and then dialyzed against DMSO/H_2O (50 vol%) for 1 day and subsequently against distilled water for 2 days. The biotinylated copolymer was recovered by lyophilization.

LCST measurement.

The turbidity of polymer aqueous solution was determined at λ = 600 nm by a Shimadzu UV-2450 UV−visible spectrophotometer equipped with a temperature control unit. The polymer concentration was kept at 0.2 wt%. The LCST was defined as the temperature inducing a 10% decreasing in the original optical transmittance.

HABA/Avidin Assay.

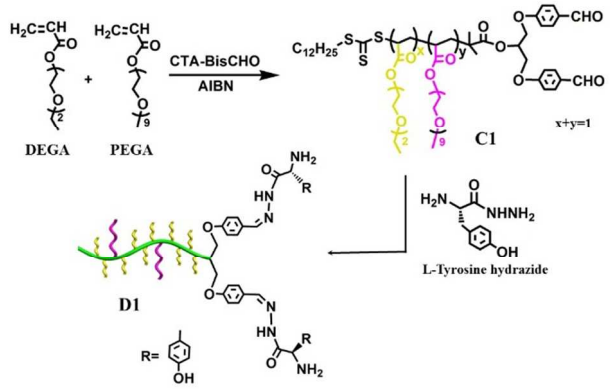
The amount of available biotin on polymer or nanoparticles was determined by an HABA/avidin binding assay. The HABA/avidin reagent was reconstituted with 10 mL of deionized water. In a 1 mL cuvette, pipet 900 μL HABA/avidin reagent and

measure the absorbance at λ = 500 nm ($A_{500}^{HABA/avidin}$) by UV–vis spectrophotometer. To this solution, 100 μL of sample was added, the solution was mixed by inversion and the absorbance at λ= 500 ($A_{500}^{HABA/avidin+sample}$) was read. The amount of the available biotin was calculated by the following formula:[31] μmole biotin/mL $= (\Delta A_{500} / 34) \times 10$, which corresponds to the μmoles of biotin per milliliter of the sample solution, where

$$\Delta A_{500} = 0.9 \times (A_{500}^{HABA/avidin}) + A_{500}^{sample\ blank} - A_{500}^{HABA/avidin+sample}$$

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE was performed with 14% polyacrylamide gels. Electrophoresis was carried out at 80 V voltage 16 mA current for 2 h. Staining was accomplished using Coomassie Brilliant Blue R-250 solution.

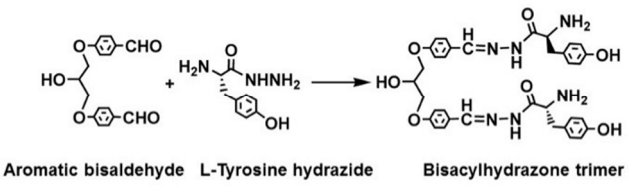


Scheme 1. Illustration of synthesis of P(DEGA-*co*-PEGA) copolymer **C1** via RAFT polymerization and tyrosine-bioconjugated dynamic covalent copolymer **D1** via acylhydrazone linkage.

Results and Discussion

Bioconjugation of Tyrosine to Thermoresponsive Copolymer

The thermoresponsive copolymer with end-functional bisaldehyde group was obtained by reversible addition-fragmentation chain transfer (RAFT) radical polymerization. In the presence of a bisaldehyde-modified RAFT agent, the copolymerization of di(ethylene glycol)ethyl ether acrylate (DEGA) and poly(ethylene glycol) methyl ether acrylate (PEGA) generated a well-defined copolymer P(DEGA-*co*-PEGA) (**C1**, M_n= 6700 Da, PDI 1.23) with bisaldehyde group at α-terminus (Scheme 1). **C1** contains 80 mol% of DEGA and 20 mol% of PEGA, displaying thermo-responsive behavior with a low critical solution temperature (LCST) value of 29.1°C.



Scheme 2. Illustration of the reaction of an aromatic bisaldehyde and L-tyrosine hydrazide.

Next, the reaction of **C1** and excess L-tyrosine hydrazide was conducted to prepare tyrosine-conjugated biodynamer **D1**. Because there are two aldehyde-reactive groups in tyrosine hydrazide: amine and acylhydrazide, the difference in the reactivity of an aromatic bisaldehyde compound towards the amine and acylhydrazide functional groups was initially investigated. With DCl as the catalyst, the reactions were performed in d_6-DMSO at 60 °C and monitored by ^{1}H NMR at specified intervals (Figures S1-S2). The reaction of the aromatic bisaldehyde and L-tyrosine hydrazide (aldehyde/hydrazide= 1:1, molar ratio) proceeded readily and the formation of acylhydrazone went to completion after 12 h (Scheme 2). However, reacting the same bisaldehyde with L-tyrosine methyl ester (aldehyde/amine=1:1, molar ratio) demonstrated that the formation of imine reached to equilibrium after 2 h, and about 60% of aldehyde reacted with amine.

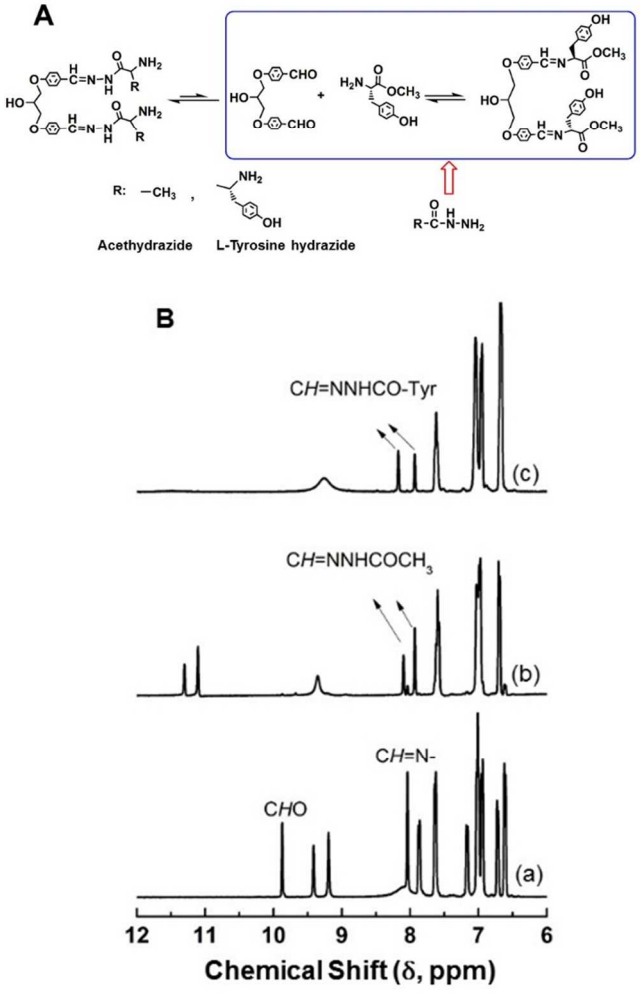


Figure 1. (A) Illustration of the reaction of an aromatic bisaldehyde and L-tyrosine methyl ester. (B) Partial ^{1}H NMR spectra in d_6-DMSO/DCl of (a) solution of an aromatic bisaldehyde and L-tyrosine methyl ester after 24 h (DCl as the catalyst), (b) 24 h after the addition of acethydrazide to the solution (a), and (c) 24 h after the addition of L-tyrosine hydrazide to the solution (a). (hydrazides/ester=1:1, molar ratio)

After the reaction of the bisaldehyde and L-tyrosine methyl ester proceeded in d_6-DMSO for 24 h, L-tyrosine hydrazide or acethydrazide was added to this solution. ^{1}H NMR analysis displayed that the signals corresponding to acylhydrazone (C*H*=NNHCOCH$_3$) appeared at 7.93 and 8.09 ppm after 24 h reaction with acethydrazide, accompanied by remarkable reductions in the intensities of aldehyde signal at 9.9 ppm and imine signal at 8.04 ppm. In the case of addition of L-tyrosine hydrazide, the signals of C*H*O and imine C*H*=N disappeared after 24 h and the signals corresponding to acylhydrazone (C*H*=NNHCO-Tyr) appeared at δ 8.18 and 7.94 ppm (*cis*+*trans*) (Figure 1). These results indicated that the exchange reaction between the formed imine and the added acylhydrazide molecule (L-tyrosine hydrazide or acethydrazide) took place and led to the formation of acylhydrazone. If amine and acylhydrazide functional groups are present in the same solution under the acidic condition employed in our study, the reaction with aldehyde group is prone to generate acylhydrazone product. Therefore, the reaction of the aromatic bisaldehyde and L-tyrosine hydrazide affords the bisacylhydrazone-linked "trimer" after the reaction reaches equilibrium under the acidic condition employed in our study (Scheme 2). Further evidence was provided by TOF-MS data, which showed the presence of a signal at *m/z*=654.36, indicating that the reaction of the bisaldehye and L-tyrosine hydrazide led to the formation of bisacylhydrazone product.

On the basis of these preliminary studies, the reaction of **C1** and excess L-tyrosine hydrazide (aldehyde/hydrazide=1:10, molar ratio) was carried out using trifluoroacetic acid as the catalyst. ^{1}H NMR analysis indicated that aldehyde group was completely consumed (Figure 2). The appearance of signals at δ 8.18 and 7.94 ppm confirmed the formation of acylhydrazone. These changes were accompanied by the disappearance of aromatic signals at δ 7.8, 7.2 ppm and the appearance of signals corresponding to the aromatic moiety of acylhydrazone at δ 7.6, 7.0 ppm. GPC analysis of the product showed that the molecular weight and PDI were almost the same as **C1** (Figure S3). The results of ^{1}H NMR and GPC analyses suggest that the copolymer and L-tyrosine hydrazide are linked through bisacylhydrazone bond to generate tyrosine-bioconjugated dynamic covalent copolymer **D1**.

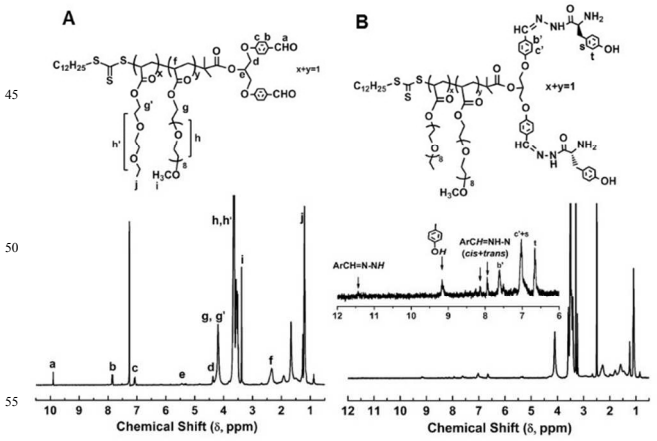


Figure 2. ^{1}H NMR spectra of P(DEGA-co-PEGA) copolymer **C1** (A, $CDCl_3$) and biodynamer **D1** (B, d_6-DMSO).

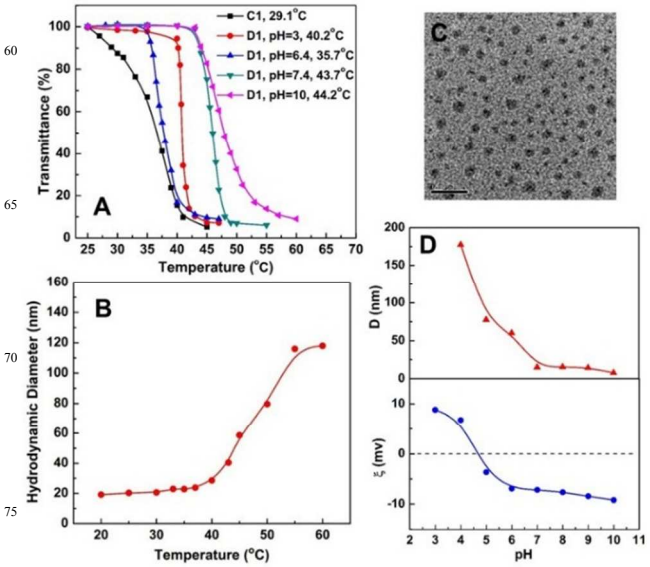


Figure 3. (A) Temperature dependence of the transmittance of **D1** aqueous solutions at various pHs. (B) Temperature dependence of the hydrodynamic diameter of **D1** in the aqueous solution. (C) TEM image of aggregates formed by **D1** at 50°C. (The scale bar represents 200 nm.) (D) pH dependence of Zeta potential and hydrodynamic diameter of **D1** at 50 °C.

After bioconjugation, the LCST of biodynamer **D1** in water (pH 6.4) shifted to a higher temperature (35.7 °C) relative to that of the precursor **C1** (Figure 3A). Dynamic light scattering (DLS) and TEM results suggested the formation of spherical micelle-like assemblies in water above its LCST (Figure 3B&C). Because values for the tyrosine amino and phenolic -OH pKa's were 9.21 and 10.46,[32] respectively, we anticipated that **D1** might exhibit pH-dependent solution properties. Having established the primary thermal response for **D1** in water, we evaluated the influence of pH on its LCST. As pH close to phenolic -OH pKa, **D1** had a higher LCST (44.2 °C) because the deprotonation of phenol group resulted in the increasing hydrophilicity of end groups. The LCST decreased to 35.7 °C at pH 6.4 due to the effect of hydrophobic phenol group. However, it was found that the LCST shifted to 40.2 °C at pH 3 because the protonation of amino group resulted in the increasing hydrophilicity of end groups. Zeta potential measurements indicated that **D1** had an isoelectric point (IEP) at pH 4.70 (Figure 3D). Therefore, the protonation/deprotonation of tyrosine amino and phenolic -OH not only switches the charge of **D1** but also alters the hydrophilic/hydrophobic balance with the change of media pH. As a consequence, **D1** possesses pH-dependent LCST values, and the size of formed assemblies above LCST also depends on the pH values. The bioconjugation of thermoresponsive copolymer with amino acid-derived component produced thermo/pH dual-responsive biodynamer.

The pH-reversible and dynamic character of the acylhydrazone linkage of biodynamer **D1** was demonstrated by incubating **D1** in the aqueous solution of pH 5 and monitoring the molecular weight by GPC at specified intervals (Figure 4). It was observed that GPC curves shifted towards higher molecular weight with time and PDI became very broad, along with the increasing in the intensity of peak corresponding to high molecular weight. This

result indicated that new polymers with high molecular weight produced. ^{1}H NMR analysis revealed the appearance of aldehyde signal (Figure S4), indicating that the acylhydrazone bonds of **D1** partially cleaved at pH 5. The constitutional reorganization arises from the pH-reversible and dynamic nature of acylhydrazone. The preliminary study on the reaction of copolymer **C1** and L-tyrosine methyl ester indicated that the imine formation took place at pH 5 (Figure S5). Therefore, the cleavage of acylhydrazone bond created a significant number of isoenergetic interchain linking of **C1** via both acylhydrazone linkage and the imine formation between amino and aldehyde, thus leading to the generation of polymers with high molecular weight. This result is in agreement with the studies of Lehn's group. They prepared polypeptide-type dynamic biopolymers by polycondensation of an amphiphilic dialdehyde and L-tyrosine hydrazide (or L-Tryptophan hydrazide) via acylhydrazone and imine formation in d_3-acetate buffer at pD 5. They proposed that the structural organization of the folded polymer chain drives the imine formation at pD 5.[7] Copolymer **C1** is amphiphilic due to the hydrophobic end groups, and the resulting dynamer tends to form aggregation structure, which enhances the stability of imine bond, driving the imine formation in the aqueous solution of pH 5.

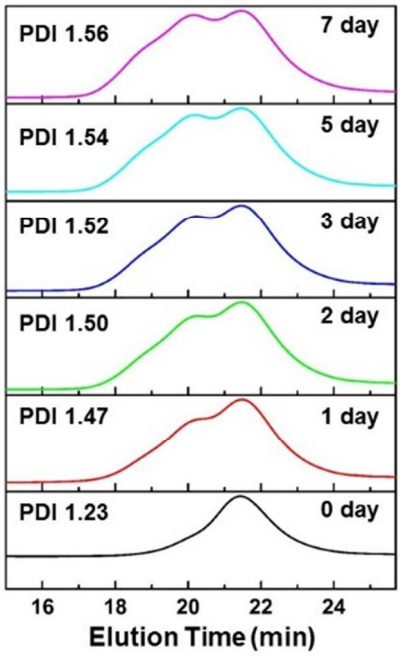


Figure 4. GPC traces of **D1** samples taken at specified intervals. **D1** was incubated in the aqueous solutions of pH 5 at room temperature.

Dynamers possess the ability to undergo changes in their constitution in response to the external environment. The intrinsic properties of the dynamic covalent polymers can be modulated by exchanging/incorporating molecular components linked by reversible covalent bonds.[5,33] The addition of 2-hydrazinopyridine (HP) to **D1** solution triggered the chain exchange reaction at pH 5.5. Because the formed bis-aryl hydrazone has a characteristic absorbance at λ 350 nm, the reaction can be monitored by UV-vis spectroscopy (Figure 5A). It was found that the exchange reaction was affected by the concentration of aniline. The absorbance at λ 350 nm increased fast with the reaction time using 50 or 100 mM aniline as the catalyst, while the absorbance changed slowly without aniline or with 20 mM aniline. The results from UV-vis indicated that aniline accelerated the exchange reaction between **D1** and HP. The copolymer obtained with 100 mM aniline was further purified by dialysis and recovered by lyophilization. ^{1}H NMR confirmed the disappearance of signals corresponding to tyrosine moiety and the presence of new hydrazone (CH=NN*H*) signals at δ 10.8 ppm attributed to HP (Figure 5B). As shown in Figure 5C, the transmittance measurement of copolymer indicated a LCST of 46.8 °C, which represented an increase of ~ 11 °C compared with the precursor **D1**, illustrating the alteration of end-group via chain exchange results in the modification of the **D1** constitution and has remarkable influence on the solution properties of the dynamer.

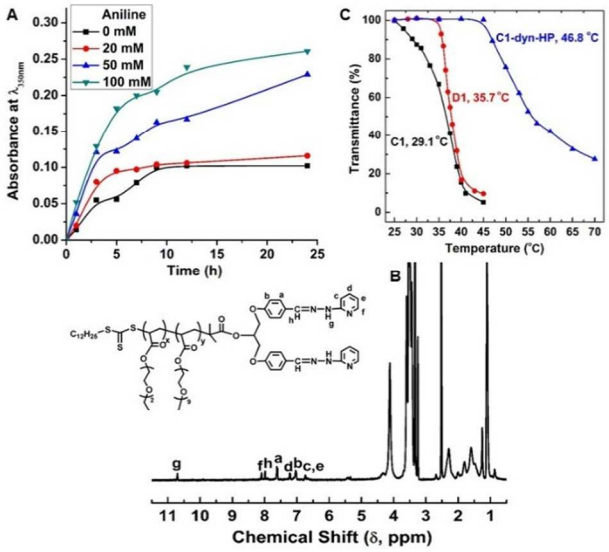


Figure 5. (A) Change of UV-vis absorbance at λ 350 nm with time during the chain exchange reactions between **D1** and HP under various concentrations of aniline. (B) ^{1}H NMR spectrum of copolymer after chain exchange reaction with HP. (C) Temperature dependence of the transmittance of the dynamer aqueous solution after chain exchange with HP.

D1 can also be endowed with biological recognition by means of chain exchange with biotin hydrazide. **D1** reacted with 5 equiv. biotin hydrazide in the aqueous solution of pH 5.5. After 24 h, the copolymer was purified by dialysis and recovered by lyophilization. The bioavailability of biotin was evaluated by an avidin/HABA competitive binding assay.[31,34] The amount of available biotin to avidin was calculated to be 2.6×10^{-3} mg/mg copolymer (Figure 6A). The recognition of biotinylated copolymer for protein was further demonstrated by the interaction with streptavidin (SA). As shown in Figure 6B, DLS data indicated that after chain exchange the biotinylated copolymer formed particles with an average diameter of 134 nm in the aqueous solution at 25 °C. The addition of SA caused the formation of large aggregates, which was attributed to the SA-

induced cross-linking of particles.[35] Therefore, it is possible to modulate and tune the biological properties of dynamic covalent polymers by chain exchange with biomolecules. Because biotin moiety was attached to the end of **C1** via reversible hydrazone linkage, the aggregates formed by SA and biotinylated copolymer were pH-responsive. As the pH was lowered to pH 5, the dissociation of large aggregates was observed because of the pH-triggered cleavage of biotin from the end of copolymer.

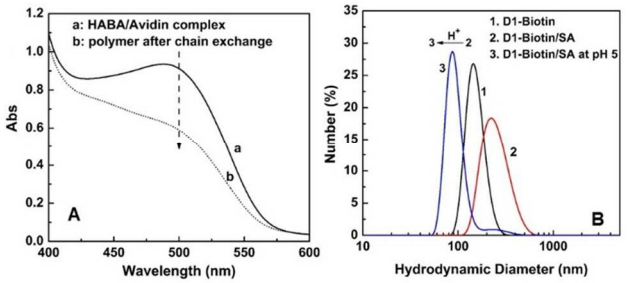
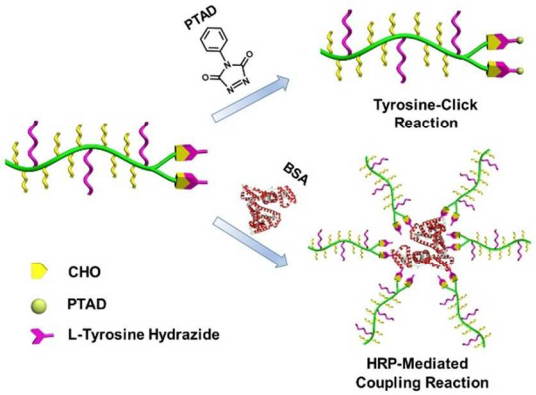


Figure 6. (A) UV-vis spectra of the HABA/avidin complex before (a) and after the addition of the copolymer obtained by the chain exchange reaction of **D1** with biotin hydrazide (b). (B) DLS curves of (1) biotinylated copolymer, (2) biotinylated copolymer/SA at pH 6.4, and (3) change the pH of solution (2) to pH 5.



Scheme 3. Illustration of the modification of **D1** by tyrosine-click reaction and HRP-mediated coupling reaction.

Modification of Tyrosine-Conjugated Biodynamer via Tyrosine-Click Reaction

Recently, Barbas and coworkers exploited the reactivity of certain cyclic diazodicarboxyamides-related molecules and the intrinsic reactivity of tyrosine, and developed a rapid aqueous ene-type reaction, the tyrosine-click reaction.[20,21] Intrigued by these results, we studied **D1** modification with cyclic diazodicarboxamides 4-phenyl-3H-1,2,4-triazole-3,5(4H)-dione (PTAD) in mixed organic/aqueous media (Scheme 3). In PBS buffer (pH 8.0)/acetonitrile (1:1), **D1** reacted with 1.1 equiv of PTAD for 24 h. After purification by dialysis, ^{1}H NMR analysis revealed the presence of the signals of acylhydrazone proton (C*H*=NNH) and aromatic –O*H*, and the appearance of signals at δ11.3, 7.42, 7.25 ppm corresponding to the modified PTAD moiety (Figure 7), confirming the attachment of PTAD to **D1** via tyrosine-click reaction. Functionalized PTAD derivatives containing azide, alkyne, maleimide, and fluorescent moiety were reported and applied to create novel antibody-drug and protein-DNA conjugates.[22] Therefore, it is expected to construct advanced biomaterials with tyrosine-conjugated biodynamer as the building block through the tyrosine-click reaction.

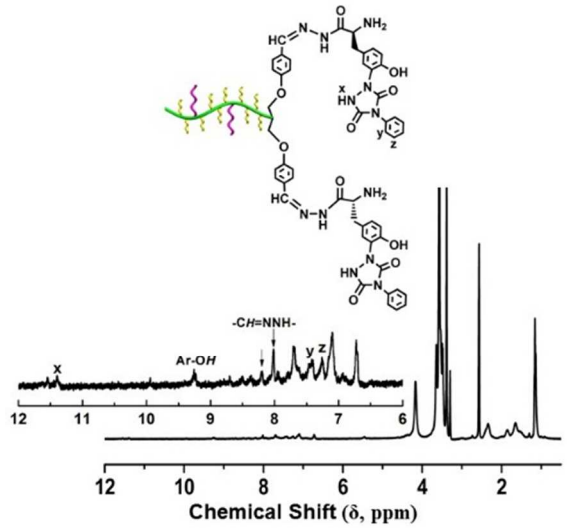


Figure 7. ^{1}H NMR spectrum of PTAD-modified **D1**.

Preparation of Protein-Dynamer Bioconjugate via HRP-Mediated Coupliong Reaction

Phenolic oxidative cross-linking has been widely observed in structural proteins such as resilin, fibroin, elastin and collagen.[36-39] Oxidoreductases, including tyrosinase, laccase, and peroxidase, can catalyze the oxidation reaction of tyrosine and form *o*-quinone and phenoxy radicals in the side-chain.[40-42] These activated tyrosine species are subsequently conjugated with other tyrosine residues. Tyrosine-mediated protein modification by the enzymatic reaction was employed for the protein-protein heteroconjugation[11-13] and the protein-chitosan bioconjugations.[43] Here, the peroxidase-mediated oxidative tyrosine coupling reaction for the covalent modification of **D1** with protein was examined (Scheme 3).

Horseradish peroxidase (HRP) was selected as the enzyme to catalyze the bioconjugation reaction. Bovine serum albumin (BSA) containing 20 tyrosine residues was employed as a model protein. As a control experiment, the reactivity of BSA against HRP treatment was tested in the presence of H_2O_2. BSA cannot cross-link by itself upon HRP treatment, as indicated by the SDS-PAGE analysis (Figure 8). Initiated by H_2O_2, the reaction of **D1** and BSA was carried out in 1M Tris-buffer (pH 8.0). The evidence of successful biodynamer-BSA bioconjugation was obtained by SDS-PAGE analysis. A mixture of **D1** and BSA resulted in bands only for BSA, indicating no protein-polymer bioconjugte was formed via simple physical interaction. The self-coupling of D1 was not visible, while the coupling of **D1** and BSA yielded the product with molecular weight higher than 66 kDa, confirming the generation of biodynamer-BSA bioconjugate (DBSA).

After purification by ultrafiltration, the thermo-responsive behavior of DBSA was studied by DLS (Figure 9A). Below 40

°C, DBSA had an average hydrodynamic diameter of ~10 nm, implying that DBSA existed as a unimer. A sharp increase of hydrodynamic diameter was observed above 40 °C, which meant the occurrence of micellization. Beyond 60 °C, the micellar size remained almost constant at ~93 nm. This clearly indicates the thermo-induced formation of micelles with the P(DEGA-*co*-PEGA) chains as the hydrophobic cores and BSA as the hydrophilic shells. TEM image showed that the aggregates were spherical with an average diameter of ~30 nm at 50 °C.

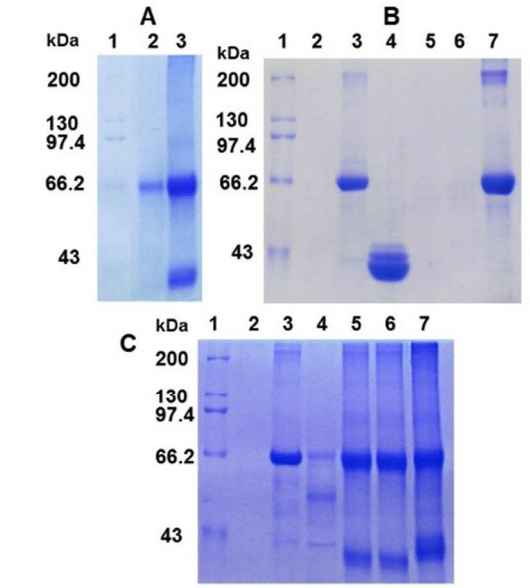


Figure 8. SDS-PAGE analyses of HRP-mediated coupling reaction of **D1** and BSA. (A) Line 2: BSA with the addition of HRP and H_2O_2; Line 3: biodynamer-BSA bioconjugate. (B) Line 3: BSA; Line 4: HRP; Line 6: **D1** with the addition of HRP and H_2O_2; Line 7: mixture of BSA and **D1**. (C) Line 3: BSA; Line 4: pH 3; Line 5: pH 4; Line 6: pH 5; Line 7: biodynamer-BSA bioconjugate. (Line 1: protein marker)

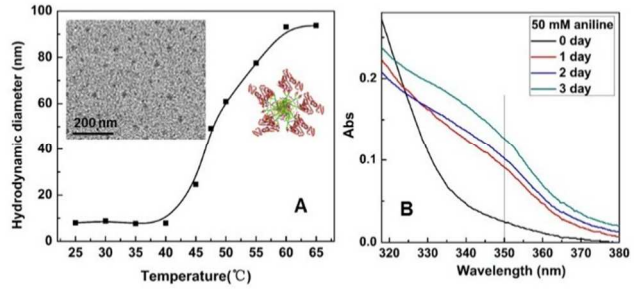


Figure 9. (A) Temperature dependence of the hydrodynamic diameter of biodynamer-BSA bioconjugate in the aqueous solution. (Inset: TEM image and illustration of aggregates formed by biodynamer-BSA bioconjugate at 50 °C.) (B) UV-vis spectra of biodynamer-BSA solution during chain exchange reaction with HP at specified intervals.

Demonstration of the dynamic character of biodynamer-protein bioconjugate was achieved by adding an excess amount of HP to the aqueous solution of DBSA at pH 5.5, and monitoring the formation of bis-aryl hydrazone bond using UV-vis. With 50 mM aniline as the catalyst, the characteristic adsorption of bis-aryl hydrazone bond gradually appeared at λ 350 nm with time (Figure 9B), confirmed the incorporation of HP into the end group of **C1** via chain exchange reaction. SDS-PAGE analysis showed that only free BSA band at 66 kDa was observed, indicating that HP replaced tyrosine hydrazide in the biodynamer, and triggered the release of BSA from DBSA (Figure S6). The pH-triggered cleavage of DBSA was accomplished under pH 3, 4 and 5 conditions. After 24 h incubation in the aqueous solution of pH 3 at room temperature, SDS-PAGE analysis showed that the band corresponding to higher molecular weight disappeared and only free BSA band was observed, which indicated the hydrazone bond between **C1** and tyrosine moiety was cleaved, thus leading to the release of BSA. While at pH 4 and 5, the hydrazone linkage was partially cleaved. These results demonstrate that the biodynamer-protein bioconjugate possesses pH-responsive and adapative features because of the incorporation of reversible hydrazone bond.

Conclusions

In summary, pH/thermo-responsive amino acid-conjugated biodynamer was generated by the bioconjugation of bisaldehyde-functionalized copolymer and L-tyrosine hydrazide via reversible covalent linkage. This biodynamer possessed a pH-dependent LCST and had an IEP value at pH 4.7. The properties of the biodynamer are tunable by constitutional modification through exchange of the terminal component, thus demonstrating the dynamic character. Because of the versatile reactivity of phenolic group in tyrosine, the biodynamer provided a reactive scaffold for conjugation. Cyclic diazodicarboxamide PTAD was attached to the biodynamer via tyrosine-click reaction. The biodynamer-protein bioconjugate generated by HRP-mediated oxidative coupling reaction possessed pH-responsive and adaptive natures. Such dynamer-protein bioconjugate combines the functional properties of protein with the reversible and stimuli-responsive characters of dynamic covalent polymers, which may have numerous applications in the areas of biotechnology and biotherapeutics.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedures of the test and corresponding ^{1}H NMR spectra, GPC curves of copolymer and biodynamer, ^{1}H NMR spectra of biodynamer in d_6-DMSO/DCl, and SDS-PAGE analysis of BSA-biodynamer after exchange reaction with HP. See DOI: 10.1039/b000000x/

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