Electronic Supplementary Material (ESI) for Polymer Chemistry

Electronic Supplementary Information

Construction of protein-crosslinked nanogels with vitamin B_6 bearing polysaccharide using the click reaction

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Materials

Pyridoxal 5'-phosphate, sodium nitrite, dimethyl sulfoxide and sodium thiosulphate were purchased from Wako Pure Chemicals (Osaka, Japan). 4-Ethynylaniline was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Sodium hydrochloride, sodium chloride, sodium L-ascorbate and copper(II) sulphate pentahydrate were purchased from Kanto Chemical Co., Ltd. (Tokyo, Japan). Pullulan (M_w, 1.0×10^5 g/mol) was purchased from Hayashibara Biochemical Laboratory, Inc. (Okayama, Japan). 1,1'-Carbonyldiimidazole and lysozyme chloride (from chicken egg white) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonate (HEPES) and dopamine hydrochloride were purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Instruments

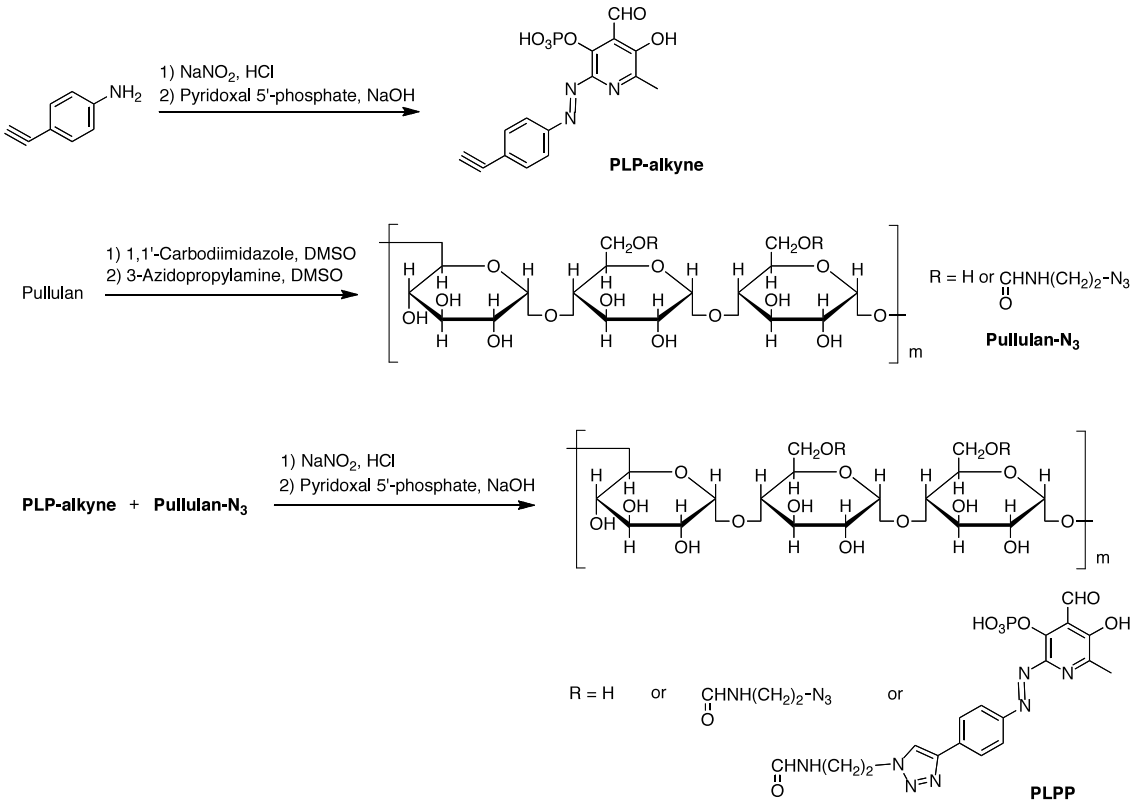
The absorbance spectra of PLPP were determined using a V-660 spectrophotometer (Jasco International Co., Tokyo, Japan) with a 2- or 10-mm path-length quartz cuvette, under thermostat control with a Peltier-effect cell holder (PAC-743R) (Jasco). The hydrodynamic diameters of PLPP/lysozyme complexes were determined by photon correlation spectroscopy (PCS) analysis using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). PCS was performed for triplicate samples at 25°C in 100 mM HEPES buffer (pH 8.0–5.0) containing 100 mM NaCl with the sampling time and analysis set to automatic. Particle size is presented as the z average of three measurements. The complexation between PLPP and lysozyme was determined by

HPLC (Tosoh Co., Ltd. Tokyo, Japan), which composed of a CCPS dual pump, a CO-8020 column oven, an RI-8020 refractive index detector and a UV-8020 UV detector. All measurements were performed using a Superdex 75 10/300 GL column with a guard column and the flow rate was 0.5 mL/min.

Synthesis of pyridoxal-bearing polysaccharide

General

Organic solvents were purified, dried and kept over a drying agent before use. All reagents were commercially available with guaranteed grades and were used without further purification. The synthetic scheme is shown in Scheme S1.



Scheme S1. Synthesis of pyridoxal 5'-phosphate-bearing polysaccharides.

Synthesis of azido-bearing pullulan (Pullulan-N_3)

The molecule was prepared from the parent alcohol by standard coupling procedures. Pullulan was first dried *in vacuo* at 70°C for 48 h, and then the hydroxyl groups of the pullulan (2.00 g, 12 mmol equivalent of anhydroglucoside units) were activated by 1,1'-carbonyldiimidazole (0.498 g, 3.08 mmol) in anhydrous DMSO (100 mL). Under a nitrogen atmosphere, the solution was stirred at room temperature for 4 h before adding 3-azidepropylamine (3.42 mL, 30.8 mmol). The solution was then stirred for 60 h at room temperature. The crude solution was placed in a Spectra/Por® 6 dialysis bag (MW cut-off = 3500, Spectrum, Houston, TX, USA) and dialyzed against distilled water and lyophilized to give a white solid (2.01 g, 95.9%). ^{1}H NMR (500 MHz, D_2O, TSP): δ1.8 (m, 2H, CH_2CH_2N$_3$), 3.3 (m, 2H, CH_2(CH_2)$_2$N$_3$), 5.0 (s, 33H per 100 glucose units, pullulan C^{1}H (1-6)), 5.4 (d, 66H per 100 glucose units, pullulan C^{1}H (1-4)). The number of 3-azidopropyl groups coupled to the polysaccharides per 100 glucose units was determined to be 6.8 based on the integrated areas of the glucopyranosyl rings and the 3-azidopropyl group.

Synthesis of pyridoxal 5'-phosphate modified with an alkyne (PLP-alkyne)

A solution of sodium nitrite (170 mg, 2.5 mmol) dissolved in water was added to a solution of 4-ethynylaniline (270 mg, 2.3 mmol) in distilled water (20 mL) and 12 N HCl (0.80 mL) at 0°C. The solution was then stirred for 75 min at 0°C. Next, pyridoxal 5'-phosphate (610 mg, 2.3 mmol) dissolved in aqueous NaOH (36 mL, pH ~10) was added drop-by-drop to the mixture. After stirring for 45 min, the pH was adjusted to ~8, and the yellow colour changed to red. The diazotization product was salted out by

adding sodium chloride. The mixture was filtered at room temperature and dried *in vacuo* to give a red solid 550 mg (61%). ^{1}H NMR (500 MHz, D_2O, TSP): δ 2.4 (s, 3H, CH_3), 5.7 (s, 2H, CH_2), 7.7 (d, 2H, J = 8.5 Hz, aromatic-H), 7.9 (d, 2H, J = 8.5 Hz, aromatic-H), 10.4 (s, 1H, CHO).

Click reaction

Three millilitres of DMSO solution containing PLP-alkyne (23.8 mg, 0.06 mmol) was added to 30 mL of DMSO solution containing pullulan-N_3 (100 mg, 0.041 mmol). Then, 10 mL of an aqueous solution of sodium L-ascorbate (84.8 mg, 0.428 mmol) and copper(II) sulphate pentahydrate (10.6 mg, 0.043 mmol) was added to the PLP-alkyne/pullulan-N_3 mixture and stirred at room temperature for 72 h. The resulting solution was placed in a Spectra/Por® 6 dialysis bag (MW cut-off = 3500, Spectrum), dialyzed against distilled water and lyophilized to yield the product (115 mg, 99.2%). ^{1}H NMR (500 MHz, DMSO-d_6/D_2O (8:2 v/v)), TMS): δ1.8 (m, 2H, $CH_2CH_2N_3$), 3.3 (m, 2H, $CH_2(CH_2)_2N_3$), 5.0 (s, 33H per 100 glucose units, pullulan C^{1}H (1-6)), 5.4 (d, 66H per 100 glucose units, pullulan C^{1}H (1-4)). The number of PLP molecules coupled to the polysaccharides per 100 glucose units was determined to be 3.8 based on the integrated areas of the glucopyranosyl rings and the 3-azidopropyl group.

Preparation of protein-crosslinked nanogels

PLPP was dissolved in buffer (100 mM HEPES, 100 mM NaCl, pH 8.0) by stirring for 1 h at 25°C. The suspension was then sonicated with a probe-type sonicator (SONIFIER

250, Branson Ultrasonics Co., Danbury, CT, USA; tip diameter, 2 mm) at 20 W for 15 min on ice and filtered through a PVDF membrane filter (MILLEX®GV, Millipore Co.; pore size, 0.22 μm) to remove dust or metallic particles shed from the titanium transducer tip. A clear aqueous PLPP solution was obtained. A given volume of 500 mM dopamine or 5 mM lysozyme solution was mixed and incubated with the PLPP solution at 25°C to yield the complex solutions.

Determination of binding constants

To evaluate the binding of dopamine or lysozyme to PLPP, UV measurements were performed (V-660 spectrophotometer, Jasco International Co., Tokyo, Japan). The UV spectra of the PLPP (0.2 mM, as a molar concentration of PLP embedded in pullulan) were measured at 25°C in the presence of varying concentrations of dopamine or lysozyme. The extent of complex formation was determined by changes in absorption at 481 nm and 399 nm for dopamine and lysozyme, respectively. The binding constants for the complexes were then evaluated by spectrophotometric titration. An iterative curve-fitting method yielded the binding constant and the maximum change in UV absorbance.

Transmission electron microscopy (TEM)

Nanogel formation by PLPP was evaluated by negatively stained TEM. Five microlitres of the PLPP solution (1.0 mg/mL) was mixed with 5 μL of an aqueous solution of phosphotungstic acid (approximately 2 wt%) on a 200 mesh copper grid deposited by

carbon, dried *in vacuo*, and washed with a small amount of water. TEM was performed using a HITACHI H-7100 (HITACHI Ltd., Tokyo, Japan).

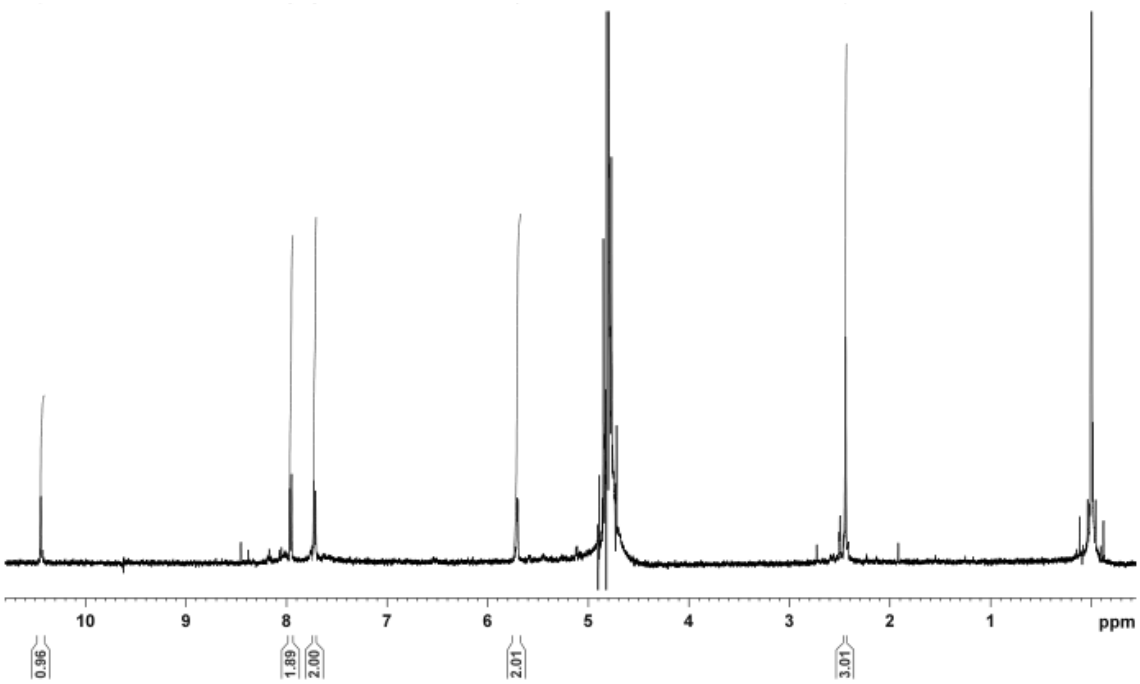


Fig. S1 ^{1}H NMR spectrum for PLP-alkyne.

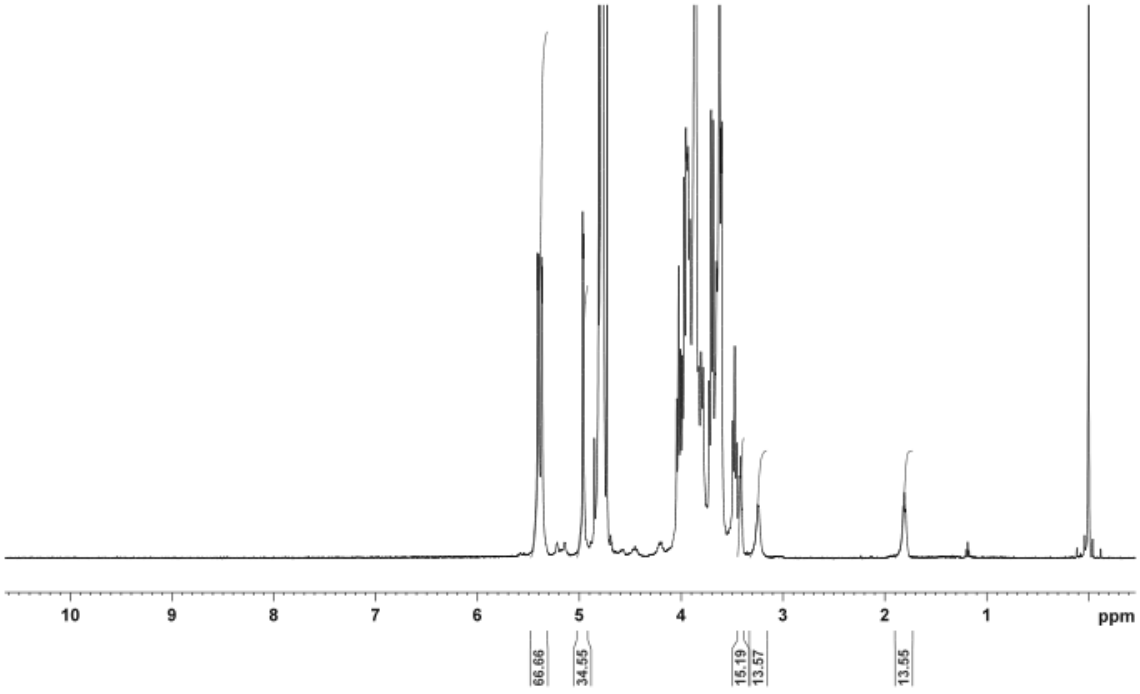


Fig. S2 ^{1}H NMR spectrum for Pullulan-N_3.

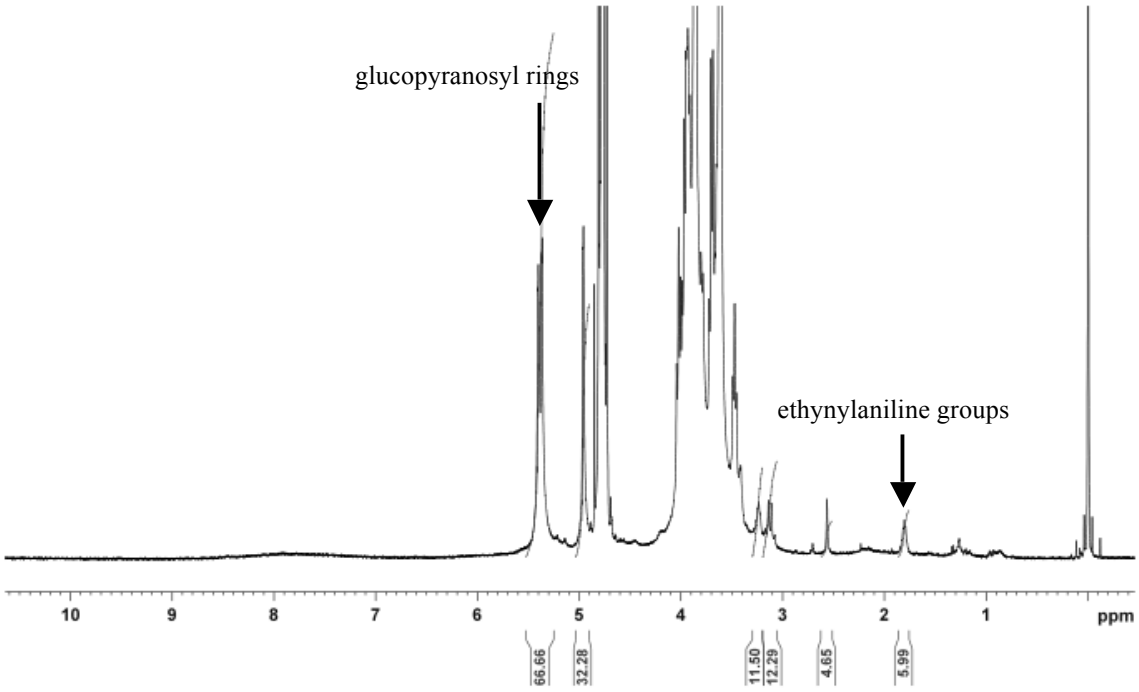


Fig. S3 ^{1}H NMR spectrum for PLPP.

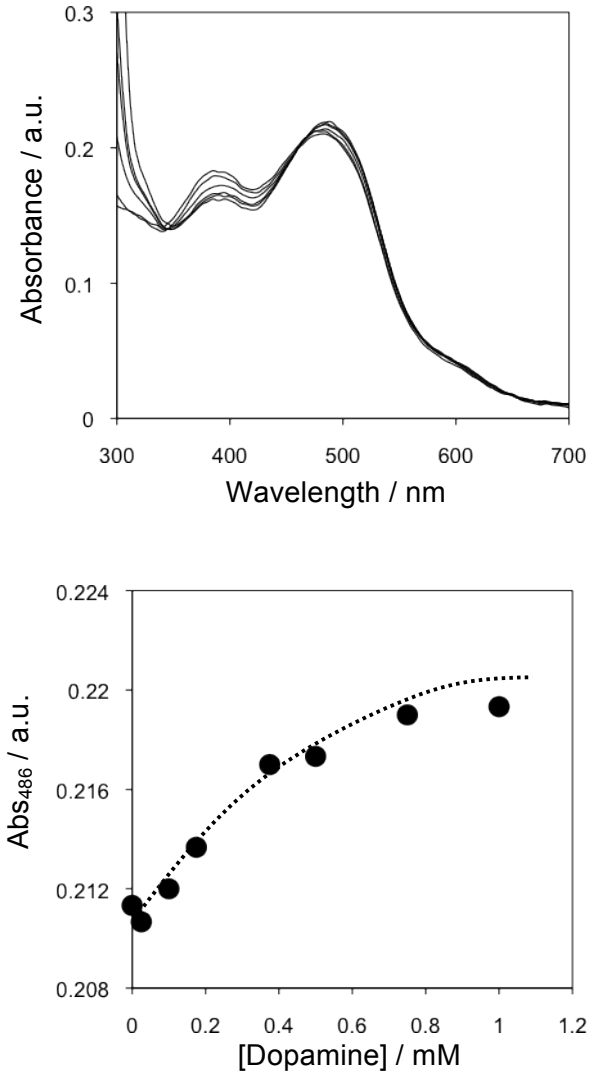


Fig. S4. a) Change in the UV spectra of PLPP (0.2 mM, as a molar concentration of pullulan-conjugated PLP) after the addition of dopamine in 100 mM HEPES buffer (pH 8.0) containing 100 mM NaCl and 50 mM $Na_2S_2O_3$ at 25°C. b) Changes in the absorbance of PLPP at 486 nm following dopamine titration.

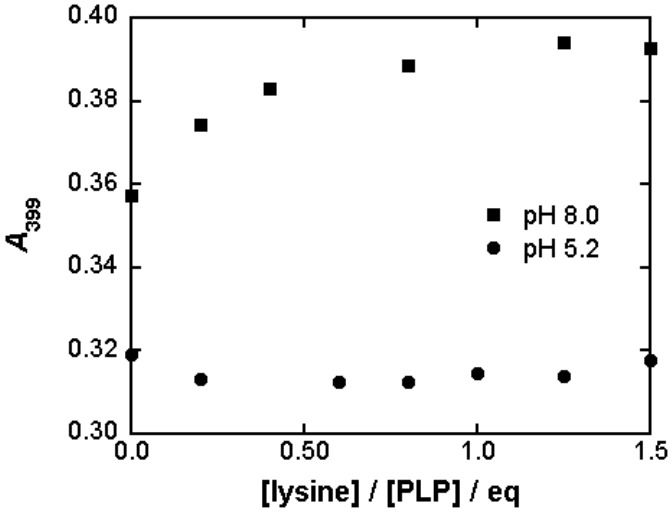


Fig. S5. Changes in the absorbance of PLPP at 399 nm following lysozyme titration at 25°C in 100 mM HEPES buffer containing 100 mM NaCl at pH 8.0 (closed square) and pH 5.0 (closed circle).

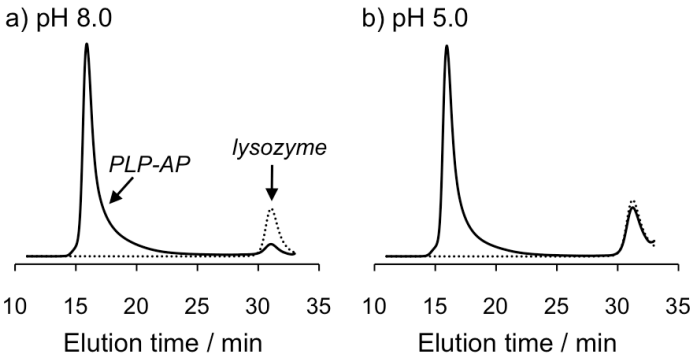


Fig. S6. HPLC elution profiles of lysozyme (0.15 mM, as a concentration of lysine residues in lysozyme) in the presence (solid line) or absence (dashed line) of PLPP (0.2 mM, as a molar concentration of pullulan-conjugated PLPP) at pH 8.0 (a) or pH 5.0 (b).