Supporting Information

Non-covalent PEGylation of proteins mediated by

site-specific \textit{in situ} polymerization induced co-assembly

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Methods

Chemicals and reagents

All chemical substances were procured from Sigma Aldrich and employed without further purification, unless stated otherwise. The synthesis of (2-(aminooxy)ethyl) 2-bromo-2-methylpropanoate (ABM) followed a previously reported protocol \(^1\). Prior to use, 2-hydroxypropyl methacrylate (HPMA) underwent purification via passage through a basic alumina column to eliminate the polymerization inhibitor. Molecular biology reagents were obtained from New England Biolabs and utilized as supplied, unless otherwise specified. Cell culture reagents and media were sourced from Gibco or Hyclone, unless stated otherwise. Daudi B cells were procured from the cell bank of the Chinese Academy of Medical Sciences. Female BALB/c nude mice, aged five weeks, were acquired from Vital River Laboratories (Beijing, China) and housed at the animal research facility of Peking University. Ethical approval for all animal experiments was granted by the Ethics Committee of Peking University (approval number LA2022274).

Synthesis of PEG-Br

5 g (1 mmol) of polyethylene glycol monomethyl ether (mPEG) with molecular weight of 5000 were dissolved in 50 milliliters of dichloromethane, to which 300 \( \mu \text{L} \) of triethylamine (2.33 mmol) were added. 247 \( \mu \text{L} \) of 2-bromo-2-methylpropanoyl bromide (2 mmol) were dissolved in 5 milliliters of dichloromethane and added dropwise within 10 minutes. The stoichiometry of mPEG: triethylamine: 2-bromo-2-methylpropanoyl bromide was 1: 2.33: 2. The reaction mixture was stirred at room temperature for 24 hours. Upon completion of the reaction, the solvent was removed by rotary evaporation. To the resulting white solid residue, 50 milliliters of ethyl acetate were added, and the mixture was rapidly stirred at room temperature for 30 minutes to dissolve the product. The solution was filtered to remove insoluble materials, and the filter cake was washed with 25 milliliters of water followed by 25 milliliters of diethyl ether. The product obtained was a white powdery solid.

Proton nuclear magnetic resonance (\(^1\)H-NMR) spectroscopy

20 mg purified powder of PEG-Br was dissolved in deuterated chloroform, PEG-PHPMA was dissolved in deuterated DMSO, and the \(^1\)H-NMR spectra were recorded on a JEOL ECX-400 400 MHz spectrometer.

Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS)

The MWs of mPEG and PEG-Br were analyzed by matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry performed on a 4800 Plus MALDI-TOF/TOF™ Analyzer (AB SCIEX) using a nitrogen laser. For the analysis, the samples were
diluted in water to a final concentration of 1 mg/mL. The matrix was a saturated solution of sinapic acid in a 50:50 mixture of acetonitrile and water containing 0.1% trifluoroacetic acid. Sample and matrix solution were mixed 1:1, and 1 μL of the mixed solution was spotted onto the sample plate and dried in air.

**Synthesis of PEG/Mb-PHPMA**

The synthesis of Mb-Br was performed as described before with slight modification. A 50-mL Eppendorf tube was loaded with Mb (25 mL of a 100 μM solution in 25 mM phosphate buffer, pH 6.5) and pyridoxal 5'-phosphate (PLP) (25 mL of a 50 mM solution in 25 mM phosphate buffer, pH 6.5). After a brief agitation to ensure thorough mixing, the mixture was incubated at 37 °C for 12 h without additional stirring. Desalting column (Cytiva) was employed to remove unreacted PLP from the reaction mixture. The purified blend (25 mL of a 50 μM solution in 50 mM phosphate buffer, pH 5.5) underwent treatment with the ATRP initiator, ABM (25 mL of a 2 mM solution in 50 mM phosphate buffer, pH 5.5), and was allowed to stand without agitation for 12 h. Subsequently, the reaction solution underwent similar purification via desalting column.

CuCl/CuCl$_2$/HMTETA was employed as catalytic system in the ATRP reaction based on previous works and several considerations: 1) HMTETA is a commonly used ligand in aqueous-phase bioconjugate synthesis, which enables relatively controlled polymerization; 2) in the preparation system described in this paper, the buffer being used was PBS, which contains approximately 150 mM chloride ions, far exceeding the concentration of bromide ions in the system (even when using the CuBr catalytic system). Typically, a 0.5 mL PBS solution containing the obtained Mb-Br (0.01 µmol), PEG-Br (0.1 µmol) and purified HPMA (22 µmol) was introduced into a Schlenk tube. The mixture underwent nitrogen bubbling to remove oxygen. Simultaneously, a catalyst solution comprising 0.01 mmol CuCl, 0.01 mmol CuCl$_2$, and 0.04 mmol 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA) in 0.5 mL MilliQ water was deoxygenated by nitrogen bubbling in another vial. The deoxygenated catalyst solution was subsequently transferred via cannula into the first deoxygenated Schlenk tube. Sealing the Schlenk tube, the polymerization reaction ensued for 2 h in an ice-water bath before quenching by air exposure. The solutions underwent a transition from transparent to slightly opaque during the reaction, indicative of in situ PEG/Mb-PHPMA assembly formation. The assembly was additionally purified and isolated from the reaction mixture using a desalting column followed by a Sephacryl S-400 gel column (Fast protein liquid chromatography (FPLC), Cytiva). Additionally, increasing the feeding ratio between HPMA monomer and macroinitiator to 500:1 and 1000:1 could yield worm-like and vesicle-like nanostructure assemblies.

**Synthesis of PEG/IFN-PHPMA**

The synthesis of IFN-Br was performed as described before with slight modification. In brief, a blend comprising 100 mM IFN-LPETGGH$_6$, 50 mM sortase A, and 2 mM ATRP initiator (AEBM) in 50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl$_2$, pH 7.4 was left to incubate overnight at room temperature without stirring. Subsequently, the reaction mixture underwent dilution and application to a HiTrap Capto Q column (Cytiva) on an AKTA purifier. Gradient chromatography ensued with 20 mM Tris-HCl, pH 7.4 as the equilibration buffer and 20 mM Tris-HCl, 1 M NaCl, pH 7.4 as the eluent. Peaks containing the desired product mixture (IFN-
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Br) were collected, followed by exchange of the IFN-Br solution to 10 mM PBS, 10% glycerol, pH 7.4 buffer using a desalting column, and concentration via ultrafiltration.

A 0.5 mL PBS solution containing the obtained IFN-Br (0.01 µmol), PEG-Br (0.1 µmol) and purified HPMA (22 µmol) was introduced into a Schlenk tube. The mixture underwent nitrogen bubbling to remove oxygen. Simultaneously, a catalyst solution comprising 0.01 mmol CuCl, 0.01 mmol CuCl\(_2\), and 0.04 mmol 1,1,4,7,10,10-hexamethyltriethylenetetramine (HMTETA) in 0.5 mL MilliQ water was deoxygenated by nitrogen bubbling in another vial. The deoxygenated catalyst solution was subsequently transferred via cannula into the first deoxygenated Schlenk tube. Sealing the Schlenk tube, the polymerization reaction ensued for 2 h in an ice-water bath before quenching by air exposure. The solutions underwent a transition from transparent to slightly opaque during the reaction, indicative of \textit{in situ} PEG/IFN-PHPMA assembly formation. The assembly was additionally purified and isolated from the reaction mixture using a desalting column followed by a Sephacryl S-400 gel column (Fast protein liquid chromatography (FPLC), Cytiva).

\textbf{Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)}

The samples were solubilized in SDS-PAGE loading buffer supplemented with 5% (v/v) 2-mercaptoethanol and 0.5% (w/v) bromphenol blue. Subsequently, they were heat-denatured at 95 °C for 5 minutes and loaded onto a precast 12% polyacrylamide gel (comprising 5% stacking gel and 12% separating gel). Electrophoretic separation was conducted using a Mini-Protein gel apparatus (Bio-Rad) at 90-120 V for 90 minutes in a running buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS), followed by staining with Coomassie Blue.

\textbf{Gel permeation chromatography (GPC)}

The GPC system utilized two 5 µm Mixed-C columns and included a WellChrom K-2301 refractive index detector operating at 950 ± 30 nm. Additionally, a Precision detector PD 2020 light scattering detector (with scattering angles of 90° and 15°) was incorporated. The eluent, consisting of THF, contained 2.0% v/v triethylamine and 0.05% w/v butylhydroxytoluene (BHT), and operated at a flow rate of 1.0 mL·min\(^{-1}\). Calibration standards for the refractive index detector comprised a series of ten near-monodisperse poly(methyl methacrylate) samples.

\textbf{Dynamic light scattering (DLS)}

Dynamic light scattering (DLS) analysis was conducted using the Malvern Zetasizer Nano-zs90 instrument. Prior to analysis, samples underwent filtration through a 0.22 mm pore size filter. The instrument operated with a laser wavelength of 633 nm and a scattering angle of 90° at 20 °C. Data processing was performed using Zetasizer software version 6.32.

\textbf{Transmission Electron Microscopy (TEM)}

The morphology of assembly nanostructure was studied by a transmission electron microscope (TEM) with a working voltage of 100 kV (JEM-1400, JEOL). samples were deposited onto copper grids coated with 230-mesh carbon film by submerging the grids in the sample solution for 2-3 minutes. Subsequently, the grids were immersed in a 2% (w/v) phosphotungstic acid
solution for 20 seconds to enable negative staining, after which they were air-dried at room temperature.

**Circular dichroism (CD)**

CD spectra were recorded in the far UV region (190-260 nm) on Jasco J-1500 instrument. Samples were d-illuted to a concentration of 0.15 mg/mL in H$_2$O.

**Critical micelle concentration (CMC) measurement**

To prepare the diluent solution, 45 µL of a nile red stock solution (0.88 mg/ml in ethanol) was added to 100 ml of PBS solution (pH 7.4), resulting in a final concentration of 1.25 µM. For CMC measurement, the sample was serially diluted by a factor of 2 with the diluent solution. After allowing the mixtures to equilibrate for 15 minutes, 100 µL of each solution was loaded onto a 96-well plate. The fluorescence emission spectrum was then scanned for each well, and the maximum emission intensity was plotted against concentration to determine the CMC.

**Myoglobin (Mb) Activity Measurement**

The Mb activity was measured according to the protocol previously published in the literature after minor modifications. Using Mb as an example, 100 µL of 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (20 mM), 10 µL of Mb (0.17 mg/mL), and 100 µL of H$_2$O (19.4 mM) in PBS were mixed, and the absorption at 734 nm was measured every 15 s for 5 min. The obtained data (absorbance–time curve) were linearly fitted, and the slope of the curve was proportional to the activity of the protein.

**Pharmacokinetics**

The pharmacokinetics assay was conducted following previously described protocols with minor adjustments. Female BALB/c nude mice (10 weeks old) were utilized for pharmacokinetic evaluation. These mice were randomly assigned to three groups (n = 3 per group) and administered IFN, PEGASYS, and PEG/IFN-PHPMA intravenously at a dose of 1 mg IFN-equivalent/kg body weight via the tail vein. Blood samples (5 µL) were collected from the tail vein at specified time intervals (1, 5, 15, 30 min, 1, 3, 6, 24, 48, and 72 h). Following a 30-minute incubation at 4 °C, the samples were centrifuged at 4000 g for 15 minutes, and the plasma was harvested and stored at -80 °C. The concentration of IFN was measured using an ELISA assay following the instructions provided with the human IFN-alpha2 ELISA kit (PBL Interferon Source). Pharmacokinetic parameters were fitted utilizing a two-compartment model.

**In vitro antiproliferative activity**

The *in vitro* activity assessment procedures followed previously established protocols with minor adaptations$^2$. Daudi B cells were cultured in RPMI-1640 medium supplemented with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Hyclone) at 37°C in a humidified atmosphere containing 5% CO$_2$. For bioassays, cells were plated in a 96-well plate (Corning) at a density of 7500 cells per well (50 µL), and serial dilutions of the samples (ranging
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from 0.0565 to 10,000 pg/mL) prepared in culture media were added to the test wells (50 µL per well in triplicate). Background and control wells consisted of media alone and media-treated cells, respectively, with cell viability defined as 0% and 100%. Following a 96-hour incubation period, cell proliferation was assessed using the MTS assay as per the instructions of the Cell Proliferation Assay kit (Promega). Data analysis, including curve fitting and IC50 determination, was conducted using GraphPad Prism 5.0 software.

Antiviral 2′,5′-Oligoadenylate Synthetase 1 (OAS1) Measurement

Female BALB/c nude mice aged 5 weeks were randomly assigned to groups (n = 3). Each group received a single intravenous injection of either IFN or IFN conjugates at a dose equivalent to 1 mg of IFN per kg of body weight, or PBS at an equivalent volume. On the third day post-injection, mice were humanely euthanized by exposure to CO₂ to extract perfused liver tissue. Liver tissue (0.1 g) was lysed using lysis buffer (1 mL) and then centrifuged to collect the supernatant for OAS1 measurement. The level of murine OAS1 was quantified using a murine OAS1 ELISA kit (DLdevelop).

Statistical analysis

Unless otherwise specified, data are expressed as the mean ± SD. All experiments were repeated at least three times. The comparison between the two groups was performed using an unpaired two-tailed t-test. For comparison between three or more independent groups, one-way analysis of variance (ANOVA) was performed using Tukey's multiple comparisons test. Statistical analysis was performed using GraphPad Prism 7. When P < 0.05, the difference was considered statistically significant.

Data available

All the primary data supporting the results of this study are provided in the paper and its supplementary information.
Supplementary figures

**Figure S1.** Gel permeation chromatography (GPC) trace (THF eluent, refractive index detector) of PEG-Br ($M_n = 5000$, $M_w/M_n = 1.17$) and PEG-PHPMA ($M_n = 19100$, $M_w/M_n = 1.32$).
Figure S2. MALDI-TOF spectra of PEG-Br and PEG-PHPMA.
Figure S3. Full-range $^1$H NMR spectra for PEG-Br in CDCl$_3$ at 25 °C. The insets show the chemical structures of PEG-Br with full peak identifications. X (CDCl$_3$) indicates the solvent peak.
Figure S4. $^1$H NMR spectra for PEG/Mb-PHPMA in deuterated DMSO at 25 °C. The insets show the chemical structures of PEG-PHPMA with full peak identifications. The HPMA monomer is actually an isomeric mixture composed of 2-hydroxypropyl methacrylate (75%) and 2-hydroxyisopropyl methacrylate (25%). Here, all peaks expected for the PEG and PHPMA chains were observed, which confirmed the successful growth of PHPMA from PEG-Br. X (DMSO) indicates the solvent, X (water) indicates the water peak. The degree of polymerization (DP) for PHPMA block was determined to be about 97 through calculating the integration areas of chemical shifts.
Figure S5. Fast protein liquid chromatography (FPLC) trace of isolated PEG/Mb-PHPMA and unreacted Mb. The initiation efficiency of Mb-Br was determined to be 73% according to the ratio of two peak areas.
**Figure S6.** Photographs of three PEG/Mb-PHPMAs with different ratios of HPMA/initiator (from left to right: 1000:1, 500:1, 200:1).
Figure S7. DLS analysis of Mb, Mb-Br and PEG/Mb-PHPMA with different ratios of HPMA/initiator.
Figure S8. TEM images of representative PEG/Mb-PHPMAs with different ratios of HPMA/initiator. (a) sphere PEG/Mb-PHPMA200. (b) PEG/Mb-PHPMA500. (c) PEG/Mb-PHPMA1000. The result shows that PEG/Mb-PHPMA could *in situ* self-assemble into nanostructures with tunable morphologies from spheres to worms and then to vesicle-like morphologies under different ratios of HPMA/initiator.
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Figure S9. Critical micelle concentration (CMC) measurement of PEG/Mb-PHPMA. Obviously, there was an inflection point that indicated the CMC of PEG/Mb-PHPMA was about 0.15 μM.
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Table S1. Pharmacokinetic parameters of IFN and its conjugates.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>IFN</th>
<th>PEGASYS</th>
<th>PEG/IFN-PHPMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma concentration at zero time C₀ (mg/L)</td>
<td>0.924 ± 0.12</td>
<td>0.989 ± 0.16</td>
<td>1.73 ± 0.19</td>
</tr>
<tr>
<td>Central compartment volume of distribution V1 (L)</td>
<td>0.0216 ± 0.0048</td>
<td>0.0202 ± 0.0023</td>
<td>0.0116 ± 0.0012</td>
</tr>
<tr>
<td>Distribution half-life t₁/₂d (h)</td>
<td>0.0907 ± 0.0088</td>
<td>1.22 ± 0.092</td>
<td>1.30 ± 0.16</td>
</tr>
<tr>
<td>Terminal half-life t₁/₂b (h)</td>
<td>0.906 ± 0.079</td>
<td>45.99 ± 5.54</td>
<td>4.40 ± 0.37</td>
</tr>
<tr>
<td>Peripheral to central rate constant K21 (1/h)</td>
<td>0.901 ± 0.025</td>
<td>0.489 ± 0.046</td>
<td>0.411 ± 0.042</td>
</tr>
<tr>
<td>Elimination rate constant K10 (1/h)</td>
<td>6.49 ± 0.85</td>
<td>0.0175 ± 0.0054</td>
<td>0.204 ± 0.034</td>
</tr>
<tr>
<td>Central to peripheral rate constant K12 (1/h)</td>
<td>1.02 ± 0.0800</td>
<td>0.0777 ± 0.0023</td>
<td>0.0747 ± 0.0024</td>
</tr>
<tr>
<td>Area under curve AUC(0-∞) (10⁶ ng/L*1h)</td>
<td>0.142 ± 0.020</td>
<td>56.4 ± 4.72</td>
<td>8.48 ± 0.53</td>
</tr>
<tr>
<td>Clearance CL (mL/h)</td>
<td>141 ± 8.54</td>
<td>0.354 ± 0.034</td>
<td>2.36 ± 0.18</td>
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</tbody>
</table>

### Reference

