Supplementary Information
for
A Light Sensitive Self-Assembled Nanogel as a Tecton for Protein Patterning Materials.

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Contents

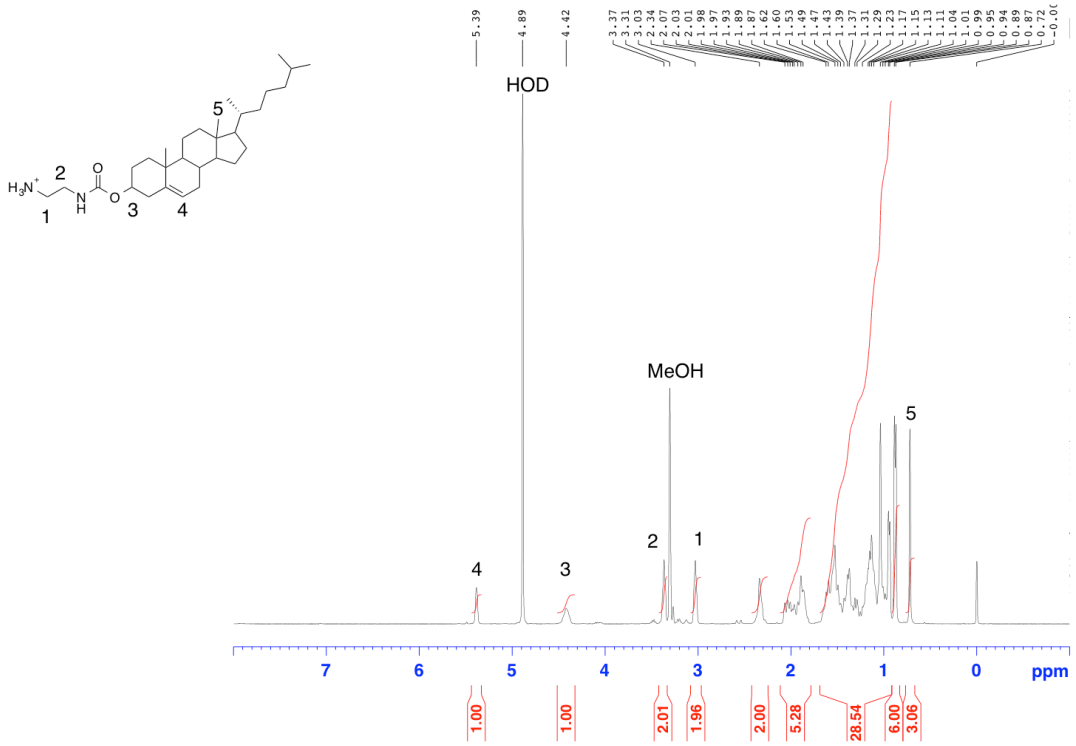
Synthesis

All starting materials were commercially available and were used without further purification. All solvents were reagent grade and were used as received. The progress of the reactions was monitored by thin layer chromatography (TLC, Merck254, silica) and the compounds were detected either by exposure to UV or by spraying with a basic solution of potassium permanganate. Flash column chromatography purifications were carried out on a 50 g SNAP ultra column using a Biotage isolera spektra. Nuclear magnetic resonance spectra were run in chloroform-d, methanol-d_4, D_2O or dimethylsulfoxide-d_6 using Bruker Avance III 400MHz spectrometers or JEOL JNM AL 400 to acquire ^{1}H and ^{13}C NMR spectra. Chemical shifts (δ) are expressed in parts per million and are reported relative to trimethylsilane (TMS) or 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt (TSP) as an internal standard in ^{1}H and ^{13}C NMR spectra.

Synthesis of 1

To a solution of *N*-boc ethylenediamine (1.08 g, 6.75mmol) and triethylamine (0.55g, 5.40mmol) in 30 mL of dry dichloromethane was added cholesteryl chloroformate (2.20g, 4.5 mmol) at 0 °C. After stirring 1 hour at room temperature, water (20 mL) was added. The organic layer was washed with water and brine, and then dried over $MgSO_4$. Evaporation of the solvent under reduced pressure afforded white powder (2.42g, 94%) The obtained solid was used without further purification.

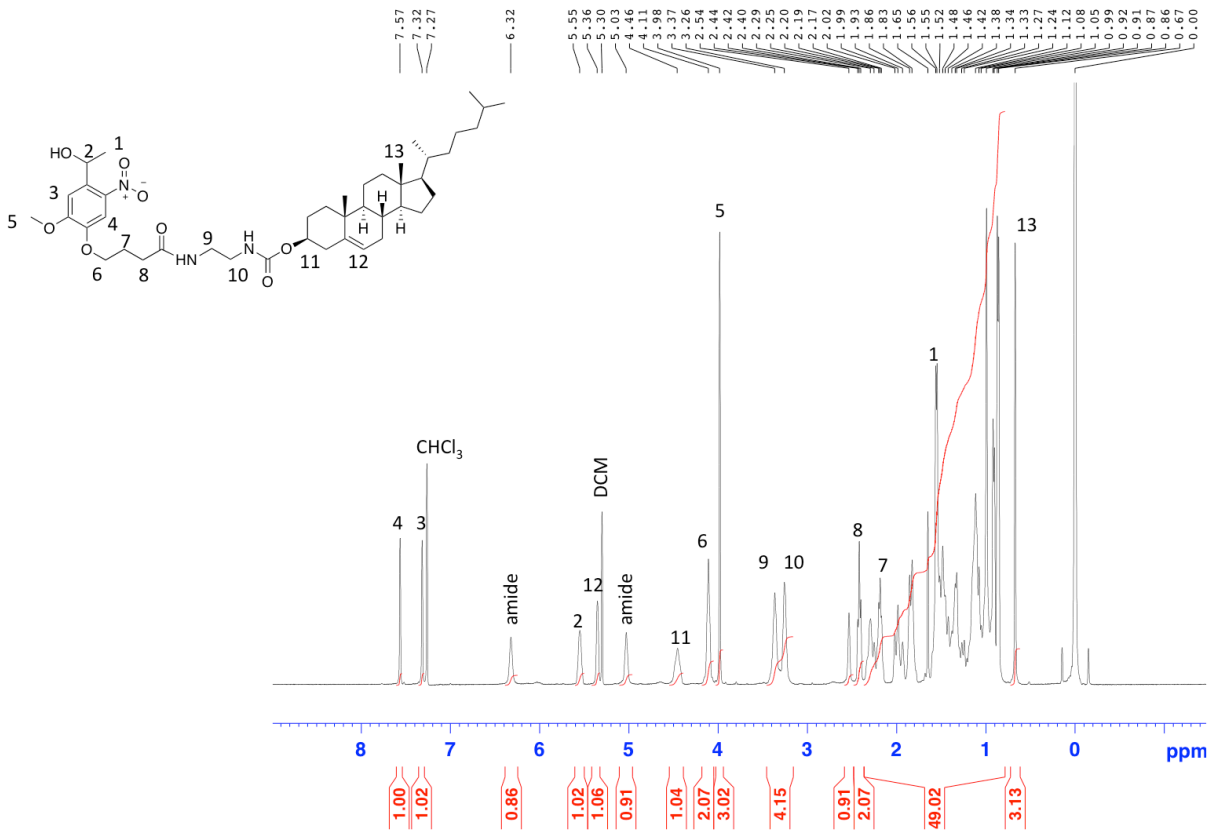
To a dichloromethane solution (20 mL) of compound 2 was added 4 M HCl/EtOAc solution. The reaction mixture was stirred for 1.5 hour for room temperature. After the reaction, the solvent and volatile materials were removed under reduced pressure. The resulting solid was washed with dimethyl ether to give 2 as a white solid (1.56g, 79%). ^{1}H-NMR (400MHz; [Methanol-d_4]): 0.76 (3H), 0.86−2.07 (51H), 2.34 (2H), 3.03 (2H), 3.37 (2H), 4.42 (1H), 5.39 (1H). ^{13}C-NMR(100.6MHz; [Methanol-d_4]): 10.9, 17.9, 18.4, 20.8, 21.6, 21.8, 23.5, 23.9, 27.76, 27.79, 27.9, 31.6, 31.8, 35.7, 36.0, 36.4, 36.9, 38.0, 38.2, 39.3, 39.7, 42.1, 50.2, 56.2, 56.7, 74.7, 122.2, 139.8, 157.7



NMR spectra of **1.** MeOH-d_4

Synthesis of 2

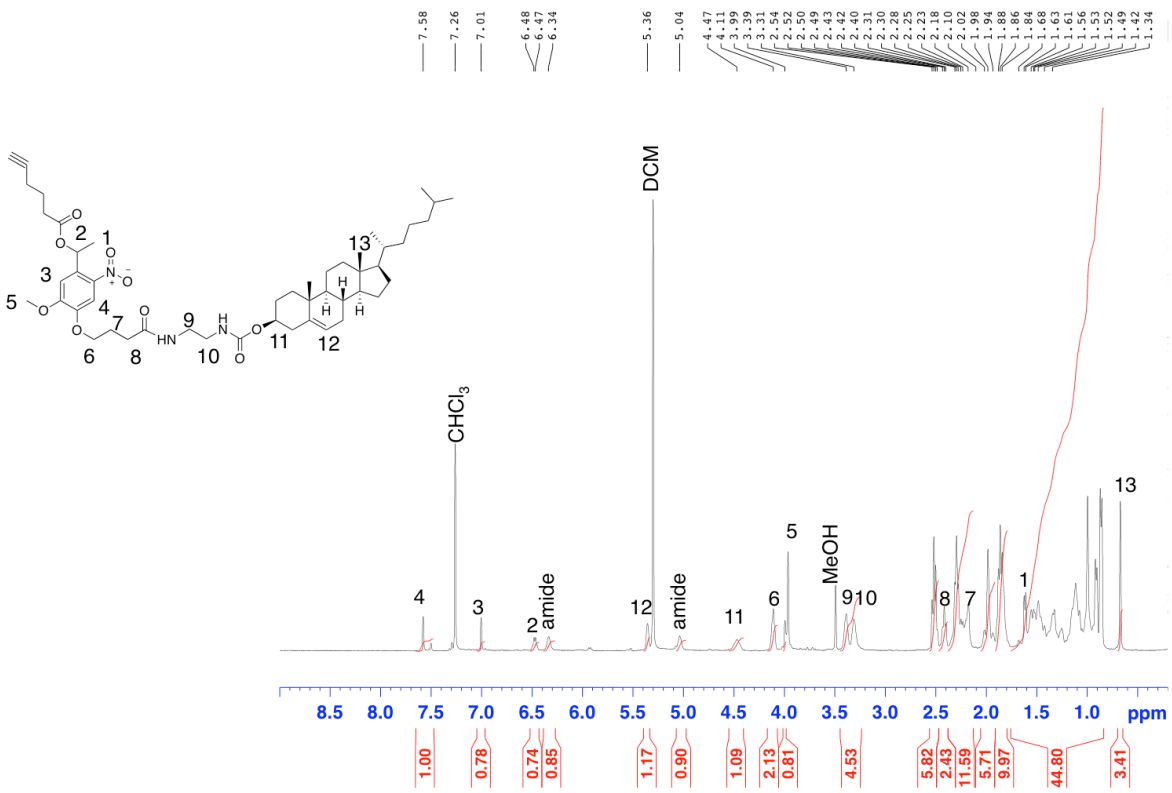
To a suspension of 1 (1.53g, 2.99 mmol), 4-[4-(1-Hydroxyethyl)-2-methoxy-5-nitrophenoxy] butyric acid (0.99g, 3.29 mmol), and triethylamine (1.24 g, 4.49 mmol) in 30 mL of dry methanol was added to DMT-MM at room temperature. After stirring 2 hours, the solvent was removed under reduced pressure. Dichloromethane (30mL) was added to the reaction mixture. The organic layer was washed with water, brine, and then dried over $MgSO_4$. The crude compound was purified with column chromatography on silica using dichloromethane: methanol = 95:5 as the eluent to give 0.88g (39%) of *o*-nitrobenzyl modified cholesterol (10,13-dimethyl-17-((*R*)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-yl (2-(4-(4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy)butanamido)ethyl)carbamate) as a pale yellow powder. ^{1}H NMR ($CDCl_3$): 0.72 (s, 3H), 0.87−2.54 (m, 52H), 3.26–3.27(4H), 3.98(3H), 4.11 (2H), 4.46(1H), 5.03(1H), 5.36(1H), 5.55(1H), 6.32(1H), 7.32(1H), 7.57(1H) ^{13}C-NMR($CDCl_3$): 11.8, 18.7, 19.3, 21.0, 22.6, 22.8, 23.8, 24.3, 24.8, 28.0, 28.1, 28.2, 31.9, 31.9, 32.8, 35.8, 36.2, 36.6, 37.0, 38.5, 39.5, 39.7 40.4, 40.7, 40.7, 42.3, 50.0, 56.1, 56.4, 56.7, 65.7, 68.6, 74.8, 108.8, 109.2, 122.6, 137.2, 139.6, 146.9, 154.0, 157.0, 172.8



NMR spectra of *o*-nitrobenzyl modified cholesterol. :$CDCl_3$

Synthesis of 2

To a solution of *o*-nitrobenzyl modified cholesterol (0.88 g, 1.17mmol), triethylamine (0.18 g, 1.76mmol) in 30 mL of dry dichloromethane was added hex-5-ynoyl chloride (0.76g, 5.8mmol) at 0 °C. After stirring 72 hour, the solvent and volatile materials were removed by evaporation. The crude compounds were purified using column chromatography on silica using dichloromethane: methanol = 96:4 as the eluent to give 0.81g(82%) of 2 as a yellow solid. ^{1}H NMR ($CDCl_3$): 0.72 (3H), 0.87− 2.52 (69H), 3.31–3.39(4H), 3.99(1H), 4.11(2H), 4.47(1H), 5.04(1H), 5.36(1H), 6.34(1H), 6.47–6.48(1H), 7.01(1H), 7.58(1H). ^{13}C NMR($CDCl_3$): 11.9, 17.8, 18.7, 19.3, 21.1, 22.1, 22.6, 22.8, 23.4, 23.5, 23.8, 24.3, 24.8, 28.0, 28.1, 28.2, 31.9, 31.9, 32.5, 32.8, 32.9, 35.8, 36.2, 36.6, 37.0, 38.5, 39.5, 39.7, 42.3, 56.1, 56.4, 56.7, 68.4, 68.6, 69.3, 69.3, 77.3, 74.8, 83.1, 108.1, 109.0, 122.6, 133.3, 139.7, 139.9, 147.1, 153.9, 157.2, 171.8, 173.2, 178.1



NMR spectra of **2**.: $CDCl_3$

Synthesis of azide functionalized pullulan.

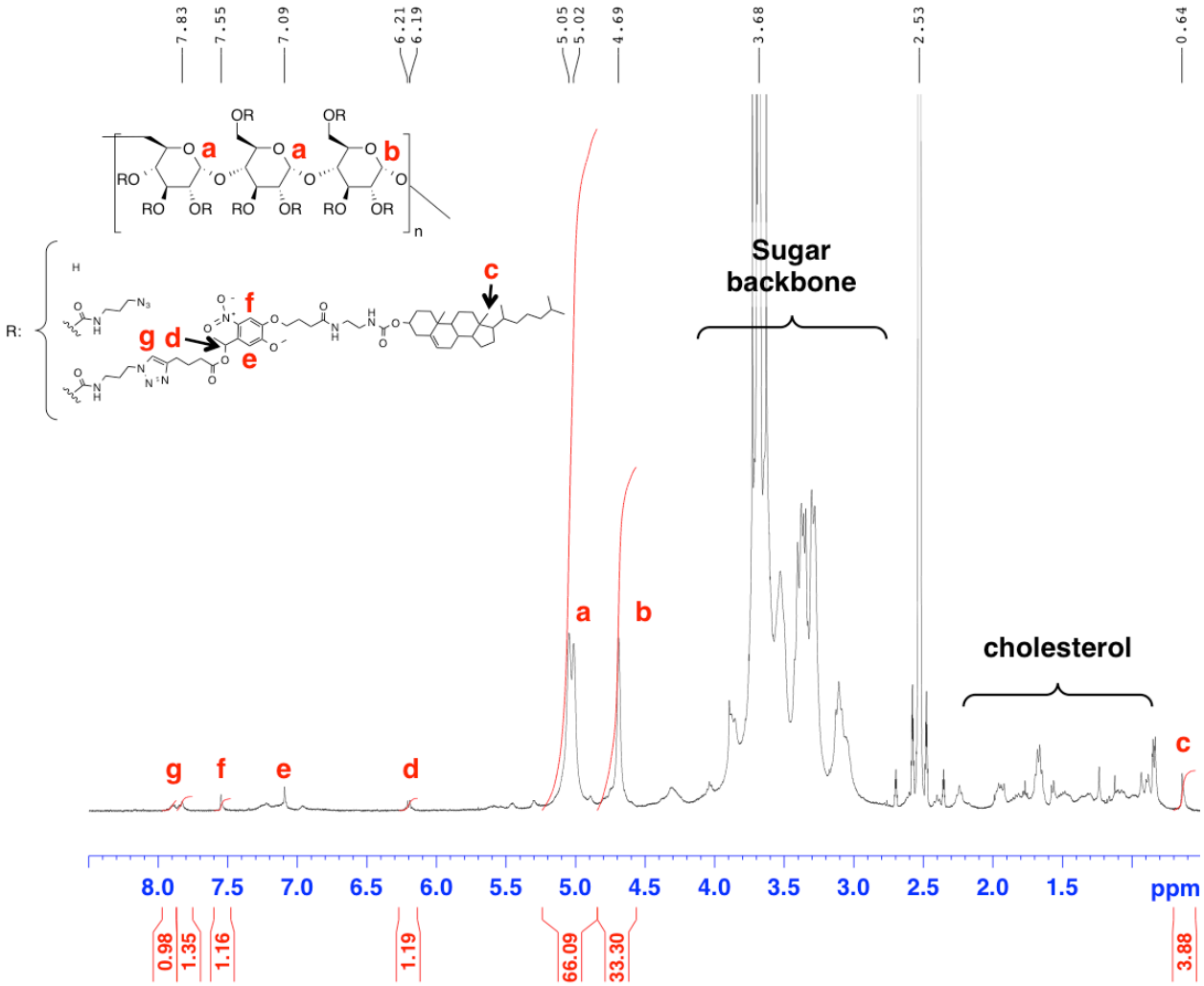
This compound was synthesized according to literature procedure.[S1]

[S1]: Biomacromolecules 2013, 14, 56−63

Synthesis of Ls-CHP.

A mixture of **2** (0.068 g, 0.08 mmol), copper sulfate pentahydrate (0.023 g, 0.09 mmol), sodium ascorbate (0.092 g, 0.46 mmol) and azide functionalized pullulan (0.50 g, 3.08 mmol) in dry *N, N*-dimethylformamide (50 mL) were stirred at 50 °C for 15 h under N_2. The reaction mixture was cooled to RT. The reaction mixture was poured into a mixture solution (500 mL) of diethyl ether : ethanol = 9.5:0.5. The resulting precipitate was collected and was washed with the diethyl ether/ethanol solution. The precipitate was dissolved in DMSO (50 mL). The

solution was dialyzed against distilled water in a dialysis membrane (molecular weight cutoff 3500, spectra por 7) for 7 days, and lyophilized to yield pale yellow powder (443mg). ^{1}H NMR (9:1 DMSO-d_6/D_2O, v/v): 0.64−2.28 (cholesterol H), 2.87−4.20 (m, glucose unit 2H, 3H, 4H, 5H, and 6H), 4.62−4.84 (br, glucose unit 1H(1−6)), 4.87−5.22 (br, glucose unit 1H (1−4), 6.19–6.21(m, methine), 7.09(s, aromatic), 7.55(s, aromatic), 7.83(m, triazole).



NMR spectra of Ls-CHP. :d_6-dmso:D_2O=9:1

Methods & Methods

Preparation of Ls-CHP solutions.

Ls-CHP was dissolved in PBS at 1.0 or 5.0 mg/ml. The solution was stirred overnight to form self-assembled particles. The solution was sonicated with a BRANSON SONIFITER MODE 1450D for 6 min, and then was centrifuged at 20,000 g for 30 min. Finally, the supernatant was filtered through a 0.22 μm filter (PVDF, MILLEX-GV Millipore) to obtain Ls-CHP solutions.

Characterization of Ls-CHP.

Dynamic light scattering (DLS) and Transmittance electron microscopy (TEM)

DLS: DLS measurements were carried out with a Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, U.K.) operating at a wavelength of 632.8 nm and a 173° detection angle.

TEM: 5 μL of above nanogel solutions were placed on a copper grid coated with an elastic carbon film. The excess sample solution was sucked away by a filter paper. A 5 μL of 2 wt% phosphotungstic acid solution as the staining agent was added and removed again. The sample was dried in a desiccator. The grid was placed in a HT-7700 (Hitachi, Tokyo, Japan) electron microscope operated at 100 kV.

SEC-MALS

SEC was performed on a chromatography system using a refractive index detector (Optilab T-rEX, Wyatt technology) connected to multi-angle laser light scattering (MALS) detector (DAWN HELEOS II, Wyatt Technology). Sephacryl S-500 10/300 was used as the column for SEC-MALS measurements. Nanogel solutions (1.0 mg/mL) were eluted with pH 7.4 PBS buffer with a flow rate of 0.50 mL/min at 25 °C. The molecular weight (*Mw*) was determined using ASTRA software based on Zimm's equation.

FFF-MALS

FFF/MALS measurement was performed by using an Eclipse 3+ separation system (Wyatt Technology, Germany) connected to a Dawn Heleos II multiangle light scattering (MALS) detector, a Optilab rEX DSP differential refractive index (RI) detector and a Dynapro

Nanostar DLS instrument with a channel flow rate of 0.5 ml/min and an isocratic cross-flow rate of 0.75 ml/min. A Wyatt channel (Eclipse 3 channel SC) was used, which has a tip-to-tip length of 18 cm and a nominal thickness of 250 mm, and a membrane (Polyether Sulfone membrane 5kDa) was attached on the bottom of the channel. The angular dependence of scattered light intensities was analyzed using Zimm's plot to determine the weight averaged molar mass.

Photo-degradation of Ls-CHP.

General procedure for monitoring photo-degradation by ^{1}H-NMR.

For the photo irradiation experiment, REX-250(Asahi spectra, japan) with a 365 nm optical band-pass filter (Bandwidth: 11 nm) was used. Nanogel solution (5.0 mg/ml) in a mixture solution of DMSO-d_6 in quartz cuvettes were irradiated with 365 nm UV light (17 mW/cm^2) for 10 and 20 min.

General procedure for monitoring photo-degradation by UV-vis spectroscopy.

Nanogel solution (1.0 mg/ml) in DMSO or PBS (1×) in a quartz cuvette were irradiated with 365 nm UV (17 mW/cm^2) light for various time interval (1, ,2, 3, 4, 5, 6, 7, 8, 9, 10, and 20 min). Each solution was characterized with UV-vis spectrometer (JASCO, V660)

General procedure for monitoring photo-degradation by SEC.

Nanogel solution (1.0 mg/ml) in PBS(1×) in a quartz cuvette were irradiated with 365 nm UV light for 10 min. The resulting solutions were characterized with DLS and SEC.

SEC data were obtained using a shimadzu prominence HPLC system equipped with a UV–vis detector (Model SPD-20A), a refractive index detector (Model RID-10A) and a column oven. KW405 (shodex) column was connected with the HPLC systems. Samples (5 mg mL^{-1}) dissolved in PBS were injected (20 mL) at a flow rate of 0.65 mL min^{-1}. SEC elution was monitored by UV absorption(350 nm).

Complexation of FITC-insulin.

Fluorescein-labeled insulin (FITC-insulin, 20 mM) was dissolved in PBS. Ls-CHP (1.0 mg) was dissolved in FITC-insulin solution at pH 7.4. After stirring for various time interval (1 h, 2h,

4h, 8h, and 24 h), the mixtures were characterized by SEC. In this case, superose 12 (GE healthcare) was connected with the HPLC system and SEC elution was monitored by UV absorption at 494 nm.

Preparation of Nanogel-based film and Photo Patterning.

Ls-CHP or CHP nanogel solution (20mg/ml) was prepared as described above. For the preparation of nanogel films, silicon sheet with a hole (ϕ= 8 mm, thickness=1.5 mm) was put on a glass slide. The nanogel solutions were poured into the hole and then dried at 40°C for overnight. The resulting film was exposed to UV light at 365 nm (17 mW/cm^2) through a PET sheet mask (TY771, 3M) for 50 min and treated with FITC-insulin solution (30 mg/ml) for 30 min. The glass slide was washed with double distilled water and was observed with fluorescence microscopy (Keyence, BZ-X700).

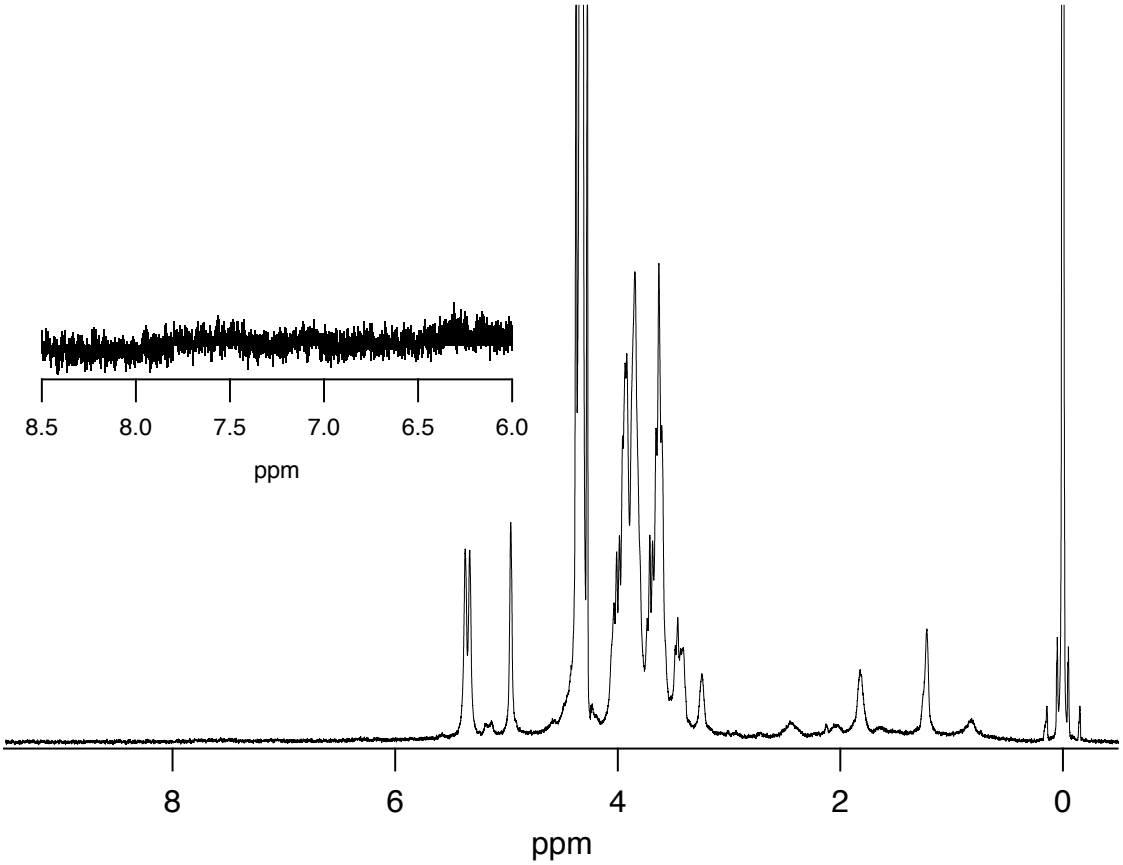


Fig. S1 ^{1}H-NMR spectra of Ls-CHP (2 mg/ml) in D_2O : 60°C, 512 scans.

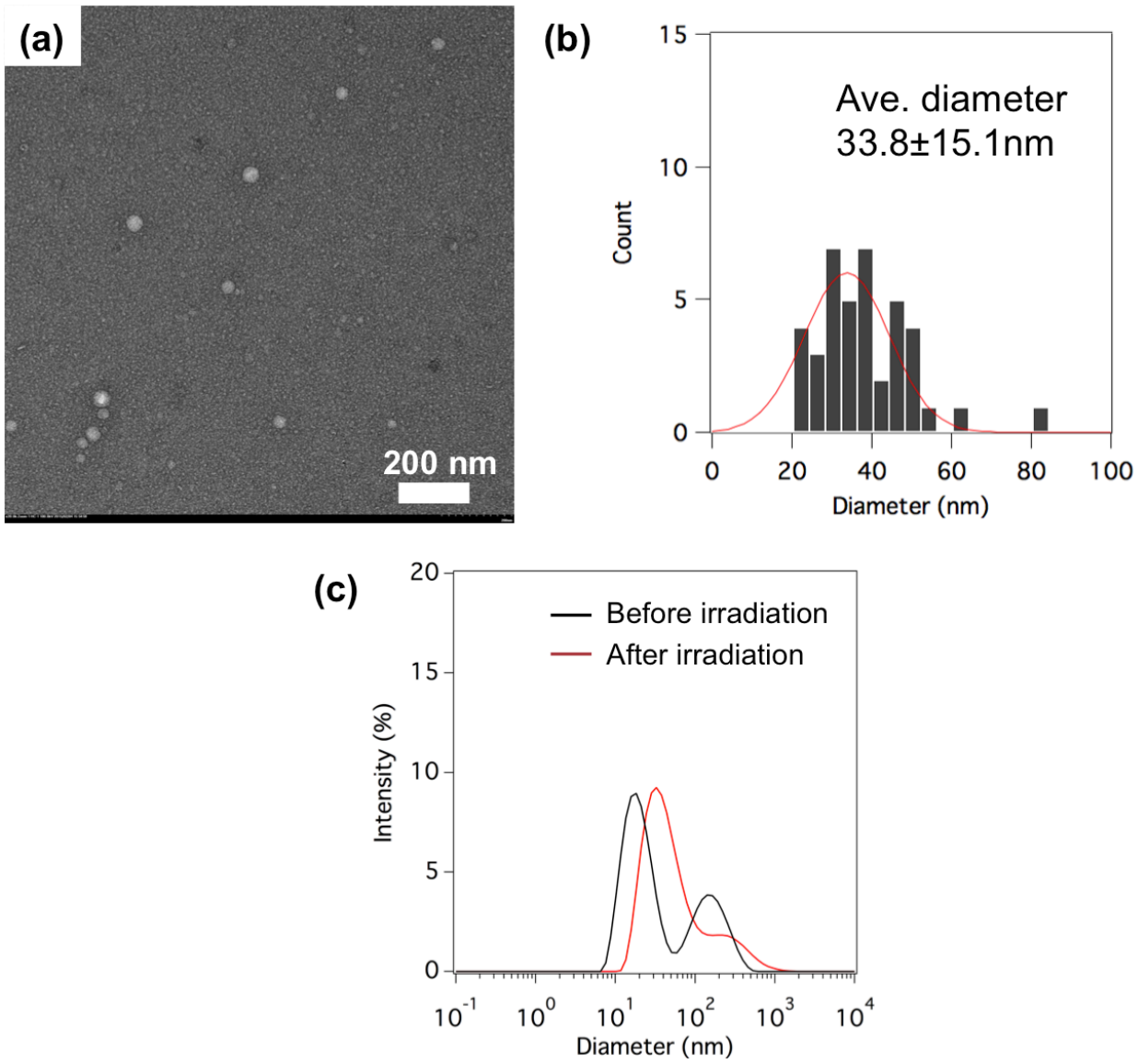
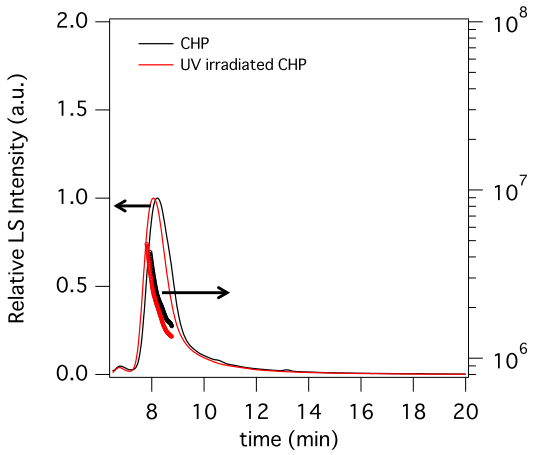


Fig. S2 (a) TEM image and (b) size distribution obtained from TEM images of Ls-CHP after 10 min photo irradiation. (c) size distribution of Ls-CHP nanogels in PBS buffer before and after photo irradiation.



Sample	M_w (g/mol)	R_w (nm)	R_h (nm)
CHP	2.1×10^6	30.8	21.8
UV irradiated CHP	2.0×10^6	32.2	23.4

Fig. S2 Field flow fractionation (FFF) chromatogram of non-cleavable cholesteryl-pullulan (CHP) and UV-irradiated CHP in water ([CHP]=2.5mg/ml). Solid line, 90° light-scattering signal; open circle, molar mass.

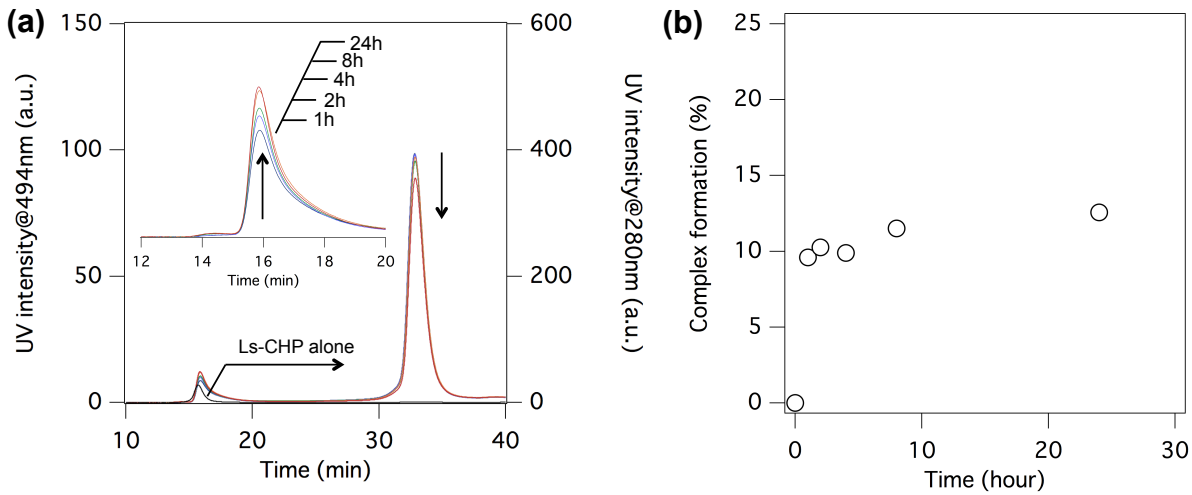


Fig. S4 Complex formation between Ls-CHP and FITC-insulin. (a) GPC chromatogram of Ls-CHP and the mixture solution of Ls-CHP and FITC-insulin as a function of time (0 – 24 h) (b) Degree of complex formation between Ls-CHP and FITC-insulin.

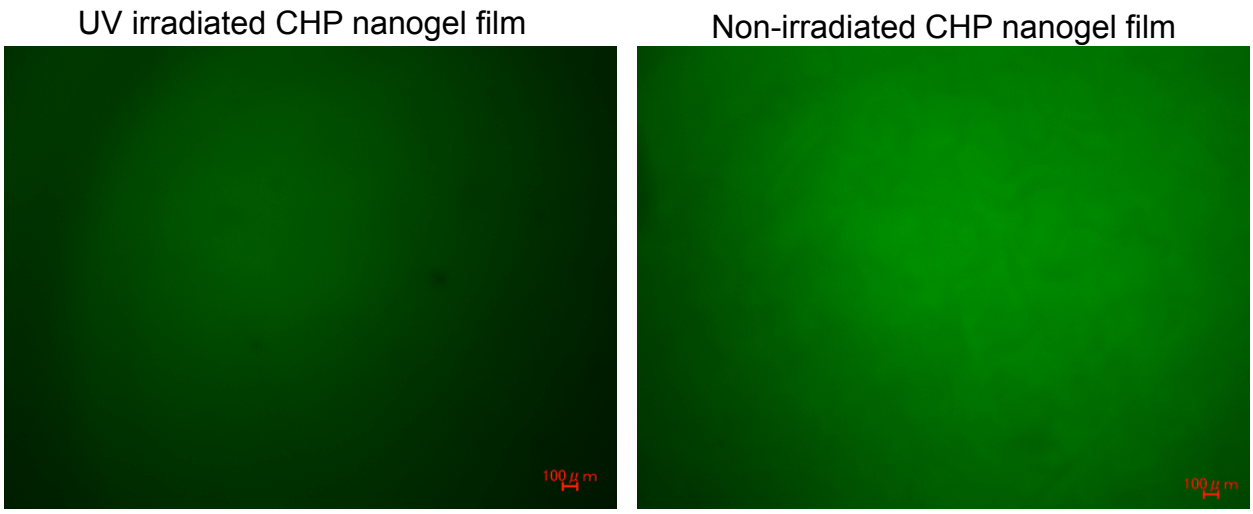


Fig. S5 Fluorescence microscopy images of non-photodegradable cholesteryl pullulan (CHP) based nanogel film. [Left: After UV light irradiation for 10 min (UV light was irradiated for all over surface of the film).