Supporting Information

Programmable Protein-DNA Hybrid Hydrogels for the Immobilization and Release of Functional Proteins

Yuzhou Wu[1†*], Chuang Li[2†], Christina Förtsch[3], Felix Boldt[1], Holger Barth[3], Yanran Wang[1], Seah Ling Kuan[1], Thuy Tam Tran[1], Viktoria Mikhalevich[1], Zhongqiang Yang[2], Dongsheng Liu[2*], Tanja Weil[1*]

1. Institute of Organic Chemistry III, Macromolecular Chemistry and Biomaterials, University of Ulm, Albert-Einstein-Allee 11, D-89081 Ulm, Germany.

2. Key Laboratory of Organic Optoelectronics & Molecular Engineering of the Ministry of Education, Department of Chemistry, Tsinghua University, 100084, Beijing, China.

3. Institute of Pharmacology and Toxicology, University of Ulm Medical Center, Albert-Einstein-Allee 11, D-89081 Ulm, Germany.

Table of Contents:

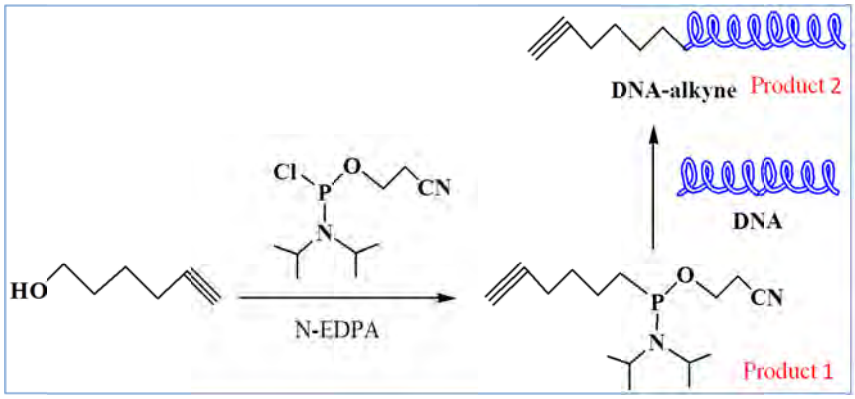
1. Materials and Instruments

Albumins from human serum (HSA) (>=98%), deoxyribonuclease I from bovine pancreas

(DNase I, lyophilized powder, protein ≥85 %, ≥400 Kunitz units/mg protein) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), ethylene diamine, calcein-AM (BioReagent, suitable for fluorescence, ⩾96.0% (HPLC)) and propidium iodide were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). Trypsin from bovine pancreas (high purity, endotoxin-free Calbiochem®) was ordered from Merck Millipore (Merck KGaA, Darmstadt, Germany). Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), urea and ethylenediaminetetraacetic acid (EDTA) were obtained from Alfa Aesar (Karlsruhe, Germany). Vivaspin® ultrafiltration tubes were purchased from GE healthcare (Munich, Germany). Dulbecco's Modified Eagle Medium (DMEM) (1X) liquid (high glucose), Fetal Bovine Serum (FBS) Standard Quality (EU approved), penicillin/streptomycin solution (100X) were purchased from PAA Laboratories GmbH (Cölbe, Germany). MEM (non-essential amino acids solution 10 mM (100X)) was purchased from Invitrogen Life Technologies GmbH (Darmstadt, Germany). CellTiter-Glo® Luminescent Cell Viability Assay was obtained from Promega GmbH (Germany). Maleimide modified DNAs (maleimide-SE3´and maleimide-SE4´) were synthesized by Gene Link (Hawthorne, NY, USA). Quick Change II XL site-directed mutagenesis was obtained from Agilent Technologies.

ÄKTA Purifier FPLC and Sephacyl S-100 HR gel filtration column from GE healthcare (Munich, Germany) was used for protein copolymer purification. Agarose gel electrophoresis was performed using Bio-Rad Mini-Sub Cell GT horizontal electrophoresis system. The MALDI-TOF mass spectrum was obtained on a Bruker Reflex™ III MALDI-TOF spectrometer. Rheological tests were carried out on an AR-G2 rheometer (TA Instruments).

2. Synthesis of Alkyne-SE1 DNA



Scheme S1. Preparation of alkyne-SE1.

5-Hexyn-1-ol (0.2 ml, 1.8 mmol) and ethyldi*iso*propylamine (0.45 ml, 2.7 mmol) were poured into 10 ml anhydrous THF and stirred, followed by addition of 2-cyanoethyl N,N-di*iso*propylchlorophosphoramidite (0.48 ml, 2.16 mmol). The reaction mixture was stirred for 1 h at room temperature followed by filtration. The filtrate was quenched with 10 ml saturated NH_4Cl. After drying in vacuum, the product was dissolved in CH_2Cl_2 and extracted with water for three times and saturated sodium chloride for two times. Then, the product was dried with anhydrous sodium sulfate and filtered and the filtrate was eluted with CH_2Cl_2:THF=1:1. The eluent was dried in vacuum to yield alkyne-phosphoramidite (product 1) as yellow oil.

Alkyne-DNA was synthesized by alkyne-phosphoramidite using a DNA solid-phase synthesizer (ABI 394) with a standard phosphoramidite DNA synthesis protocol. The CPG loaded DNA was synthesized in 1 μmol scale with a "DMT-on" method and then cleaved by concentrated ammonia solution at 60 °C for 3 h, followed by removal of ammonia using a concentrator. The primary crude product was purified by HPLC using TEAA buffer (triethylamine and acetic acid buffer, 100 mM, pH 7.0) as eluting solvents. The product was characterized by MALDI-ToF.

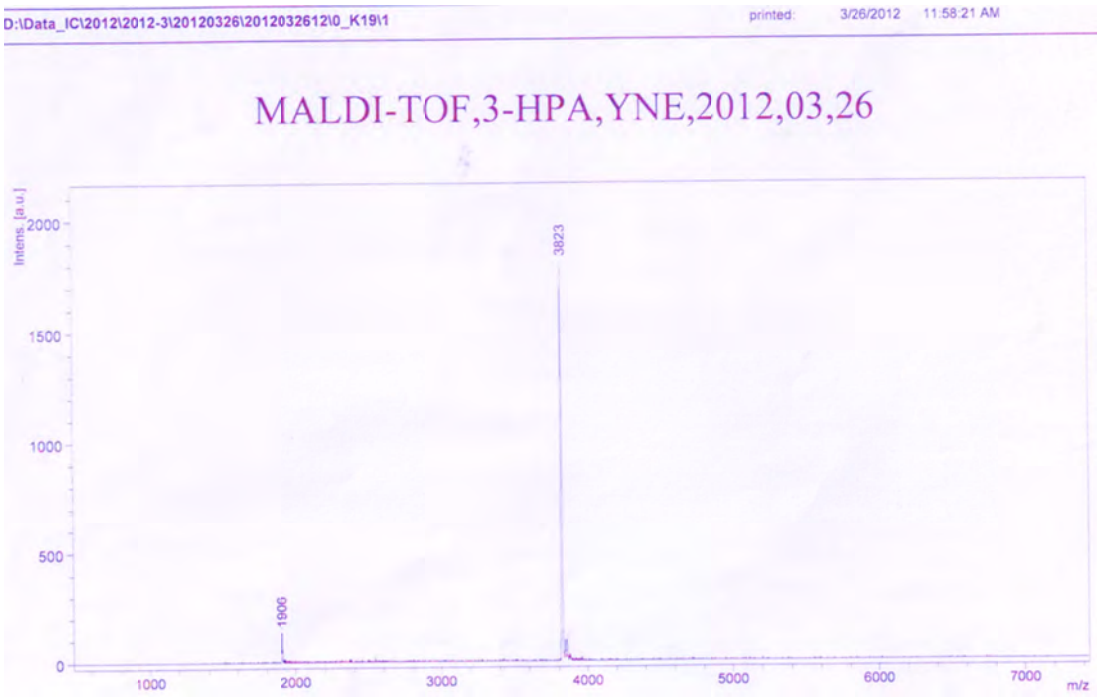


Figure S1. MALDI-ToF spectrum of alkyne-SE1, found Mw of 3823 g/mol (calculated Mw of 3821 g/mol).

3. Preparation of cHSA (2)

HSA (600 mg, 9.03 µmol) was dissolved in 60 mL ethylenediamine solution (50 mM, pH=4.75) at RT. Thereafter, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC, 3.067 g, 16 mmol) was introduced. After stirring for 2 hours at room temperature, the reaction was stopped by adding acetate-buffer (4 mL, 4 M, pH=4.75). The colorless solution was concentrated by ultrafiltration (Amicon Ultra 30 kDa MWCO), washed two times with acetate-buffer (100 mM, pH=4.75) and five times with MilliQ-water and subsequently lyophilized. cHSA (633 mg, 98 %) was isolated as white solid. MALDI-ToF: m/z 71.282 (M^+), matrix: Sinapinic acid.

4. Preparation of cHSA-PEG(2000)$_{19}$ (3)

cHSA (50.1 mg, 0.7 µmol) was dissolved in degassed phosphate-buffer (50 mL, 50 mM, pH=8.0). MeO-PEG(2000)-NHS ester (30 equivalents) were dissolved in 500 µL DMSO and then added to the reaction mixture. The solution was stirred for 135 minutes at room temperature. In order to remove unreacted PEG-containing starting material as well as cHSA, the solution was washed eight times with MilliQ-water by ultrafiltration (Amicon Ultra 30 kDa MWCO) and lyophilized. cHSA-PEG(2000)$_{19}$ (64.8 mg, 58 %) was isolated as white solid. MALDI-ToF: m/z 109.673 (M^+), matrix: Sinapinic acid.

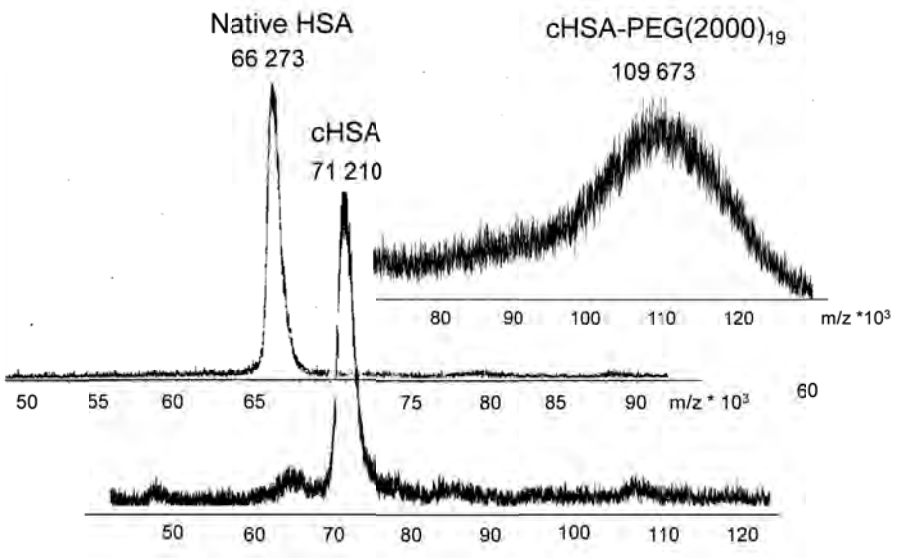


Figure S2: MALDI-ToF spectra of native HSA, cHSA and cHSA-PEG(2000)$_{19}$. According to the molecular weight increase between cHSA and native HSA, almost all 99 glutamic and aspartic acid groups of native HSA have reacted with ethylenediamine to afford cHSA with about 159 primary amino groups (60 amino groups from lysine residues and 99 amino groups from the reaction). The comparison of the molecular weights of cHSA-PEG(2000)$_{19}$ and cHSA indicates that about 19 PEG(2000) chains were conjugated.

5. Preparation of azido human serum albumin (N_3-cHSA-PEG(2000)$_{19}$, 5)

A solution of copper sulfate (102.3 µg, 400 nmol) and potassium carbonate (11.25 mg, 81.5 µmol) were mixed. The reaction mixture was then added to a solution of cHSA-PEG(2000)$_{19}$ (50 mg, 0.46 µmol) in *ca.* 2 mL of MilliQ water and thereafter, a solution of imidazole-1-sulfonyl azide hydrochloride (7.12 mg, 40.7 µmol) in water was added. The resultant purple solution was stirred for 24 hr at RT to give a brown mixture. Excess reagents were removed by ultrafiltration (MWCO 30 kDa) three times using water and subsequently freeze dried to give a white solid (40 mg), which was used as it is for the next reaction step. IR, KBr, ν (cm^{-1}): 3293 (N-H and O-H), 2927 (C-H), 2870 (C-H), 2098 (-N_3), 1649 (Amide I), 1537 (Amide II), 1452, 1249 (Amide II), 1080 (C-O-C).

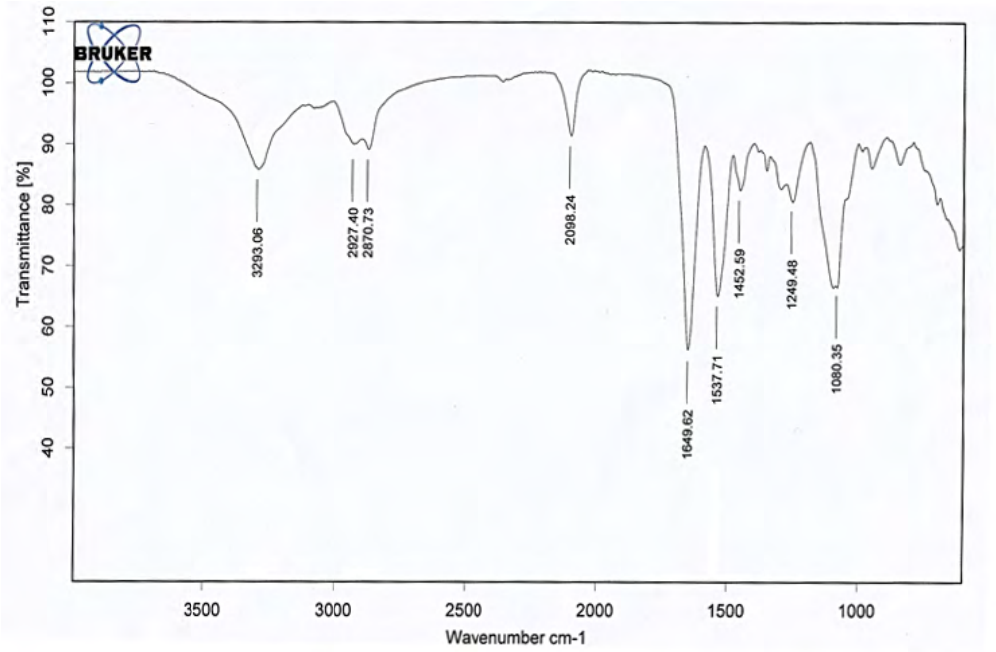


Figure S3: IR spectrum of N_3-cHSA-PEG(2000)$_{19}$.

6. Preparation of cHSA-PEG(2000)$_{19}$-SE1$_5$ (6)

N_3-cHSA-PEG(2000)$_{19}$ (12.5mg, 125nmol) was dissolved in 9 mL of PBS buffer (pH 7.4). $CuSO_4 \cdot 5H_2O$ (1.9 mg, 7.5 µmol) and sodium ascorbate (2.22 mg, 11.25 µmol) were mixed in 800 µL PBS buffer (pH 7.4) and added to a solution containing N_3-cHSA-PEG(2000)$_{19}$. Thereafter, the alkyne-SE1 stock solution (200 µL, 5mM) was added and the reaction mixture was stirred at room temperature overnight. The protein-DNA conjugate was purified by ultrafiltration (30 kDa MWCO Vivaspin 20 centrifugation membrane filter) with 50mM EDTA (pH 7) for 2 times and with Milli-Q water for 3 times, followed by gel filtration using Superdex® 200 10/30 GL column on the Äkta purifier system. The purified product was desalted by ultrafiltration in water and lyophilized to afford cHSA-PEG(2000)$_{19}$- SE1$_5$ (**6**) as white solid (15.1 mg, yield 93%).

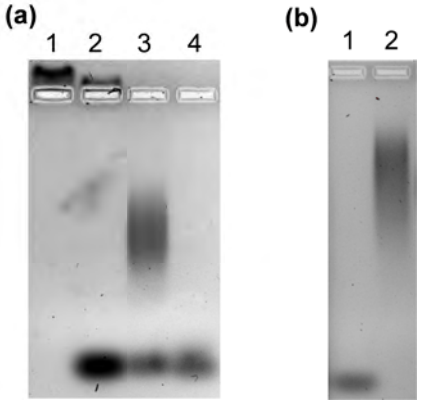


Figure S4: Agarose gel electrophoresis (1%) for DNA "click" reaction. (a). Line 1: control of pure N_3-cHSA-PEG(2000)$_{19}$ (**5**). Line 2: the mixture of N_3-cHSA-PEG(2000)$_{19}$ (**5**) and alkyne-SE1 DNA without adding $CuSO_4$ and sodium ascorbate. This control shows that non-specific absorption of SE1 DNA onto the protein does not occur. Line 3: Click reaction mixture. Since alkyne-SE1 DNA is given in excess, N_3-cHSA-PEG(2000)$_{19}$ (**5**) was completely consumed and

the formation of new protein-DNA conjugates was observed. Line 4: Control of alkyne-SE1 DNA. (b). Line 1: Control of alkyne-SE1 DNA. Line 2: cHSA-PEG(2000)$_{19}$-SE1$_5$ (**6**) after purification.

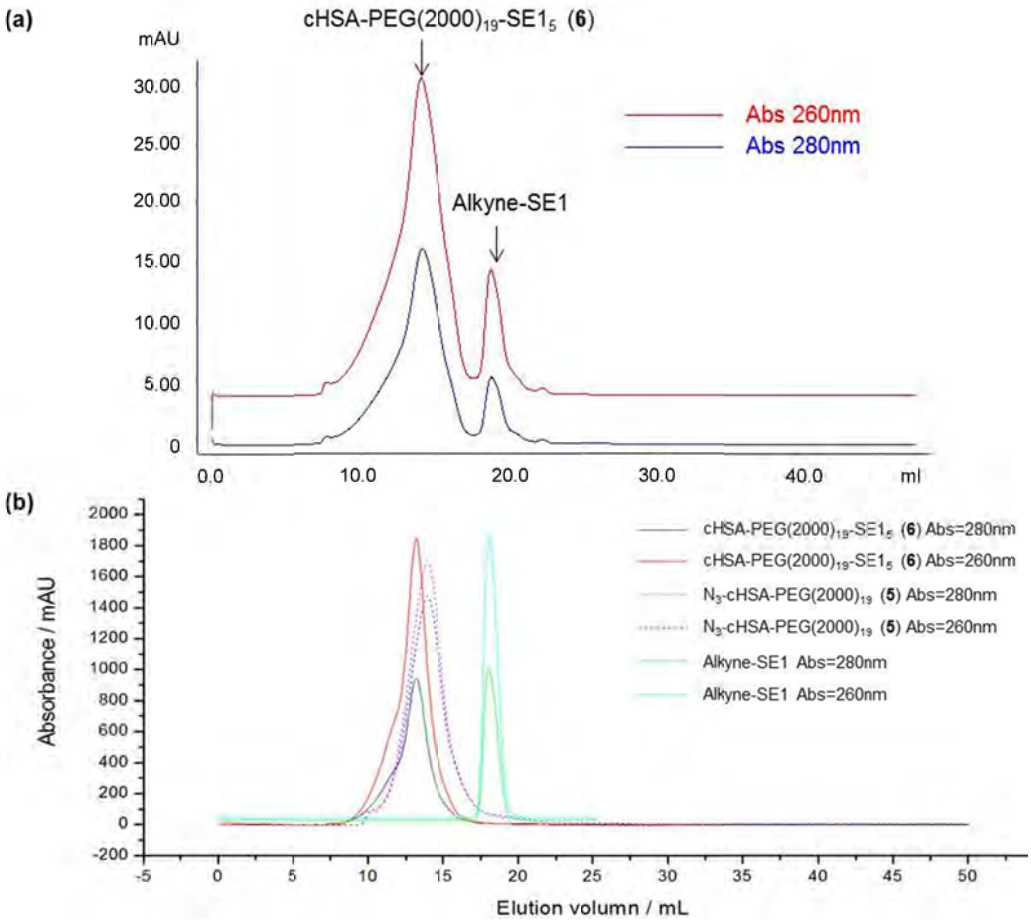


Figure S5: (a) Gel filtration chromatography of cHSA-PEG(2000)$_{19}$-SE1$_5$ (**6**) purification. (b) Gel filtration chromatography of purified cHSA-PEG(2000)$_{19}$-SE1$_5$ (**6**) compared with the standard sample of N_3-cHSA-PEG(2000)$_{19}$ (**5**) and alkyne-SE1.

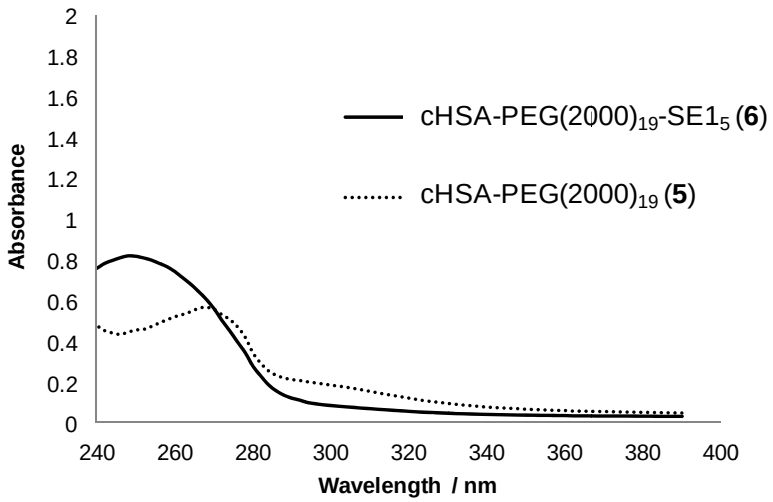


Figure S6: UV-Vis spectrum of 0.5 µM of cHSA-PEG(2000)$_{19}$- SE1$_5$ (**6**) and cHSA-PEG(2000)$_{19}$ (**5**) to estimate the number of SE1 attached to cHSA-PEG(2000)$_{19}$ (**5**) .

The number of SE1 on cHSA-PEG(2000)$_{19}$- SE1$_5$ (**6**) was calculated with the following formula:
Number of SE1 = ((A_{260} (**6**) – A_{260} (**5**))/ extinction coefficient of SE1)/ concentration of (**6**)
= ((0.82 – 0.45) / 151300 $M^{-1}cm^{-1}$) / ($0.5x10^{-6}$ M) = 5

7. Preparation of denatured HSA-derived polypeptide copolymer (PcP, 8)

Urea-sodium phosphate buffer (5mL, 5M Urea, 50mM sodium phosphate buffer, pH7.4) was degassed under Argon. N_3-cHSA-PEG(2000)$_{19}$-SE1$_5$ (14.3 mg, 110 nmol) was added to the degassed urea-sodium phosphate buffer and stirred for 15 min to allow protein denaturation. Thereafter, TCEP (tris (2-carboxyethyl) phosphine hydrochloride, 3.15 mg, 11 µmol) was added to the reaction mixture and stirred for 30 min to reduce all disulfides. N-(2-aminoethyl)maleimide (2.79 mg, 11 µmol) was then added to the solution and the reaction mixture was stirred overnight under Argon atmosphere. The product was purified by ultrafiltration (30 kDa MWCO Vivaspin 20 centrifugation membrane filter) with water for 3 times and gel filtration using Superdex® 200 10/30 GL column on the Äkta purifier system. The resulting product was again desalted by ultrafiltration with water for 3 times and lyophilized to give denatured HSA-derived polypeptide copolymer (PcP, **8**) as white solid (13.2 mg, 92% yield).

8. Amine quantification assay[1]

Fluorescamine (30 µL, 3 mg/mL) in DMSO was added to a 20 µL solution of glycine in borate buffer (pH 8.2) in varying concentrations starting from 0 mM to 1 mM and the solutions were incubated for 15 min in 384 well microplates at room temperature. The fluorescence intensity was recorded with λ_{ex} = 365 nm λem = 480 nm and the resulting linear plot (Figure S7, R^2 = 0.99) allows quantification of amine groups. All readings were obtained in triplicates.

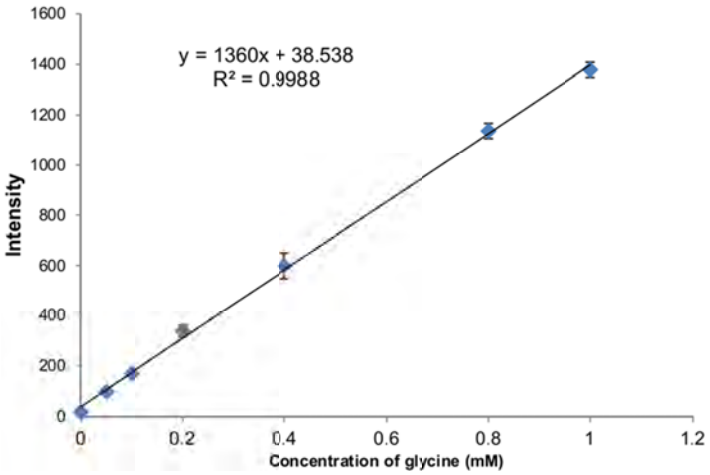


Figure S7. Calibration curve of the amine quantification assay.

Table 1. Quantification of amine groups exposed at the surfaces of HSA, cHSA, cHSA-PEG(2000)$_{19}$ and N_3-cHSA-PEG(2000)$_{19}$.

Name	No. of Amine	Theoretical No. of Amine
HSA	60 ± 1	60[1]
cHSA	127 ± 5	159[2]
cHSA-PEG(2000)$_{19}$	104 ± 2	140[3]
N_3-cHSA-PEG(2000)$_{19}$	49 ± 2	--

[1] The number of amino groups according to HSA amino acid sequence.

[2] This number is calculated by the number of ethylene diamine groups according to MALDI-ToF MS taking into account the number of amino groups of the HSA sequence.

[3] This number is calculated by the theoretical number of amnino groups in cHSA minus 19 PEG chains according to MALDI-ToF MS.

9. DNA sequences

S1-1 CCT GTC TGC CTA ATG TGC GTC GTA AGT AAC TGG ACA CTT
S1-2 CTT ACG ACG CAC AAG GAG ATC ATG AGT AAC TGG ACA CTT
S1-3 CTC ATG ATC TCC TTT AGG CAG ACA GGT CAG AGT GGA TTG
S2-1 CCT GTC TGC CTA ATG TGC GTC GTA AGC AAT CCA CTC TGA
S2-2 CTT ACG ACG CAC AAG GAG ATC ATG AGC TGT CAT CGG TCA
S2-3 CTC ATG ATC TCC TTT AGG CAG ACA GGG ACA CAC TAA GGT
Alkyne-SE1 Alkyne- AAG TGT CCA GTT
Maleimide-SE3' Maleimide-TGA CCG ATG ACA G
Maleimide-SE4' Maleimide-ACC TTA GTG TGT C

10. Preparation of 3-arm DNA linker (DL1).

S1-1, S1-2 and S1-3 (100 µL from 100 µM stock solution) were mixed in 1 x TBE buffer, heated at 95 ^{o}C for 1 min and then incubated at room temperature with gentle shaking overnight. After self-assembly, the solution was concentrated to 2 mM of **DL1**. This solution was directly used for preparing the hydrogel and it should be consumed within one week for stability reasons.

11. Preparation of DL1+Y2 4-arm DNA linker (DL2)

S1-1, S1-2 and S1-3 (100 µL from 100 µM stock solution) were mixed in 1 x TBE buffer, heated at 95 ^{o}C for 1 min, and then incubated at room temperature under gentle shaking overnight to assemble **DL1**. S2-1, S2-2 and S2-3 (100 µL from 100 µM stock solution) were also mixed in 1 x TBE buffer, heated at 95 ^{o}C for 1 min, and incubated separately at room temperature with gentle shaking overnight to assemble Y2. The **DL1** and Y2 solutions were then directly mixed and incubated at room temperature overnight. The resulting self-assembled 4-arm DNA linker (**DL2**) was then concentrated to the desired concentration before usage.

12. Expression of GFP and YFP containing a single cysteine mutation

Native GFP sequence was inserted to pET-25b(+) vector as describe before[2]. Gly51 was mutated to Cysteine using Q5 site-directed mutagenesis kit (New England Biolabs). Mutated protein was expressed in BL21(DE3) E. Coli cells under 25 ^{o}C for 4 hrs. mGFP (G51C) was purified by Ni-NTA affinity column, desalted and lyophilized. The sequence of mGFP (G51C) was shown

below (single accessible cysteine is highlighted):

MSKGEELFTG VVPILVELDG DVNGHKFSVS GEGEGDATYG KLTLKFICTT CKLPVPWPTL
VTTFSYGVQC FSRYPDHMKQ HDFFKSAMPE GYVQERTIFF KDDGNYKTRA EVKFEGDTLV
NRIELKGIDF KEDGNILGHK LEYNYNSHNV YIMADKQKNG IKVNFKIRHN IEDGSVQLAD
HYQQNTPIGD GPVLLPDNHY LSTQSALSKD PNEKRDHMVL LEFVTAAGIT HGMDELGVVG
LVPRGSHMGA GPGWPHHHHH H

YFP containing a single cysteine was prepared by inserting a cysteine containing sequence at N-terminal. The mYFP was expressed using pET-22b(+) vector in BL21(DE3) E. Coli cells under 30 ^{o}C for 4 hrs. mYFP was purified by Ni-NTA affinity column, desalted and lyophilized. The sequence of mYFP was shown below (single accessible cysteine is highlighted):

CCTSICVSKG EELFTGVVPI LVELDGDVNG HKFSVSGEGE GDATYGKLTL KFICTTGKLP
VPWPTLVTTF GYGLQCFARY PDHMKQHDFF KSAMPEGYVQ ERTIFFKDDG NYKTRAEVKF
EGDTLVNRIE LKGIDFKEDG NILGHKLEYN YNSHNVYIMA DKQKNGIKVN FKIRHNIEDG
SVQLADHYQQ NTPIGDGPVL LPDNHYLSYQ SALSKDPNEK RDHMVLLEFV TAAGITLGMD
ELYKKLAAAL EHHHHHH

13. Preparation of GFP-SE3 and YFP-SE4 conjugation.

Maleimide SE3 (350 µL of 100 µM stock solution in water) and mGFP (350 µL of 100 µM stock solution in water) were mixed in PBS buffer (total volume of 1 mL) and incubated under gentle shaking for 4 hrs. The reaction mixture was directly purified by gel filtration chromatography using Superdex 200 column on the Äkta purifier system. The fractions were further analyzed by 10% agarose gel electrophoresis and only the pure GFP-SE3 conjugates were collected and used in the following experiments. The concentration of GFP-SE3 was calculated based on GFP fluorescence. YFP-SE4 was prepared following the same procedure as described for GFP-SE3.

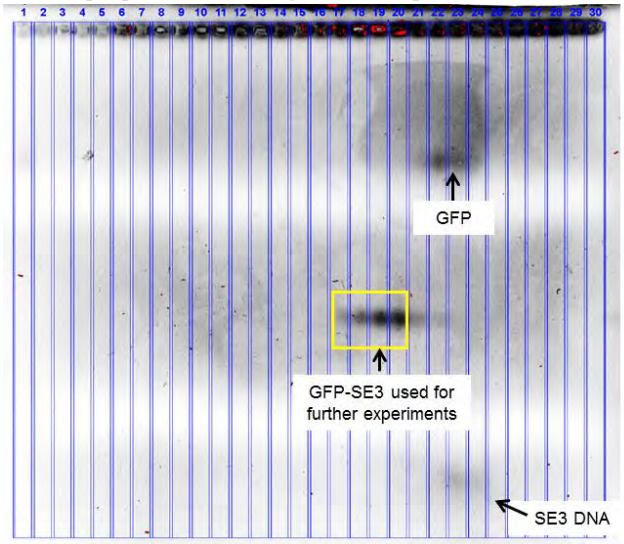


Figure S8. Gel electrophoresis analysis of all fractions after gel filtration chromatography of GFP-SE3 purification.

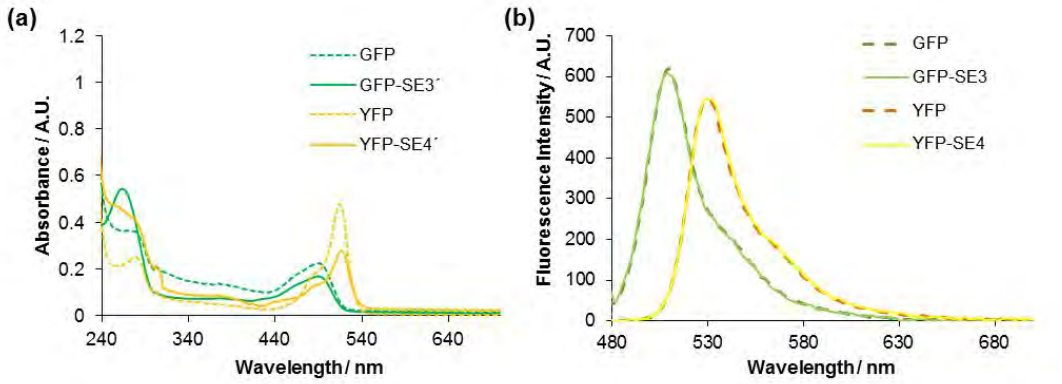


Figure S9. (a) UV-Vis and (b) fluorescence spectra of GFP, YFP, GFP-SE3 and YFP-SE4.

14. DNA-programmed assembly of GFP and YFP with DL2

One equivalent of GFP-SE3 and one equivalent of YFP-SE4 were added to the **DL2** solution in 1x TBE buffer and incubated overnight at 4 ^{o}C. The resulting solution was concentrated to the desired concentration before usage.

15. Preparation of the protein-DNA hybrid hydrogel

Denatured HSA-SE1 conjugate was dissolved in Milli-Q water to a weight percentage of 15%. To prepare the hydrogel with different solid contents, the desired amounts of denatured HSA-SE1 in solution were mixed with 5x TBE buffer and Milli-Q water. Then, the desired amount of 2 mM **DL1** in solution was added and the solutions were subsequently well mixed. Gelation proceeds within 1 min. As an example, the preparation of 50 µL 5% hydrogel required denatured HSA-SE1 in solution (15%, 11.26 µL), which was mixed with 5x TBE buffer (10 µL) and Milli-Q water (17.46 µL). Then, the **DL1** solution (2 mM, 11.26 µL) was added to the protein solution and both were immediately mixed under vigorous stirring. A transparent hydrogel was formed within 1 min. To prepare the hydrogel containing GFP/YFP, **GFP/YFP-DL2** should be premixed with the **DL1** linker at a desired ratio (e.g. 5%) and then added the protein solution as described above.

16. Rheological characterization of the hydrogel formation.

Rheological tests were carried out on an AR-G2 rheometer (TA Instruments) equipped with a temperature controller. Three types of rheological experiments were performed in 8 mm parallel-plate geometry using 40 µL of hydrogels (resulting in a gap size of 0.15 mm): i) In order to find the linear viscoelastic region of the time sweeps, oscillatory strain sweep (0.01–100%) and frequency sweep (0.05 Hz-100 Hz) were conducted at 25 ºC. The linear viscoelastic region was found to be in the range of 1% strain and 1 Hz frequency; ii) Frequency sweep tests were carried out between 0.05 and 100 rad s^{-1} at 25 ºC at a fixed strain of 1%; iii) Time-scan tests were done at a fixed frequency and strain of 1 Hz and 1%, respectively, at 25 ºC for 5 min.

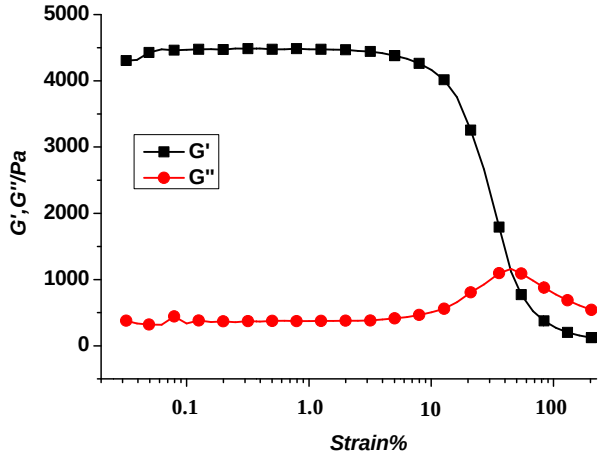


Figure S10. Oscillatory strain sweep (0.01–100%) of 5% hydrogel at 25 ºC with a fixed frequency of 1 Hz.

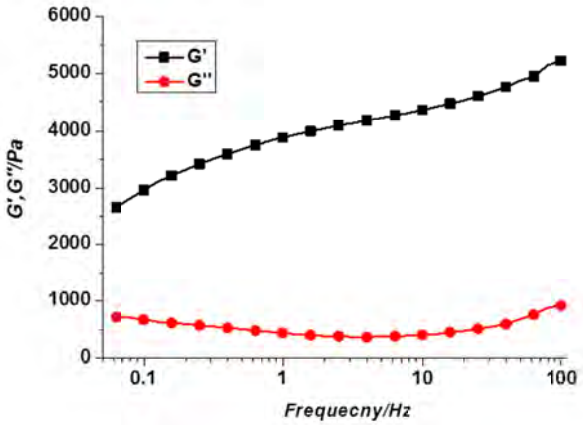


Figure S11. Frequency sweep (0.05 Hz-100 Hz) of 5% hydrogel at 25 ºC with a fixed strain of 1%.

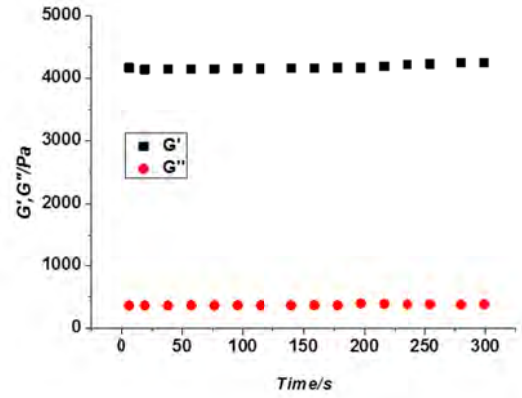


Figure S12. Time sweep of 5% hydrogel for 5 min with a fixed strain of 1% and frequency of 1 Hz at 25 ºC.

17. Circular Dichroism (CD) of the protein based hydrogel backbone (6)

Circular dichroism spectra were measured on JASCO J-810 Spectropolarimeter. cHSA-PEG(2000)$_{19}$-SE1$_5$ (**6**) was dissolved in pure water in ~0.2 mg/ml concentration. The sample was measured in 1mm cuvette with a volume of 400ul. The CD signal measured from 260nm down to 180 nm. The Bandwidth was set to 1 nm and a response at 1 sec. Standard sensitivity was used with the data pitch 0.1 nm and 100 nm/min scanning speed. Temperature was kept constant at 20 °C. The data was record by 5 times data accumulation. The data was analyzed with DichroWeb (http://dichroweb.cryst.bbk.ac.uk) using CONTIN method. 60% Helical structure, 5% beta sheet structure and 35% turns and unordered structure was obtained.

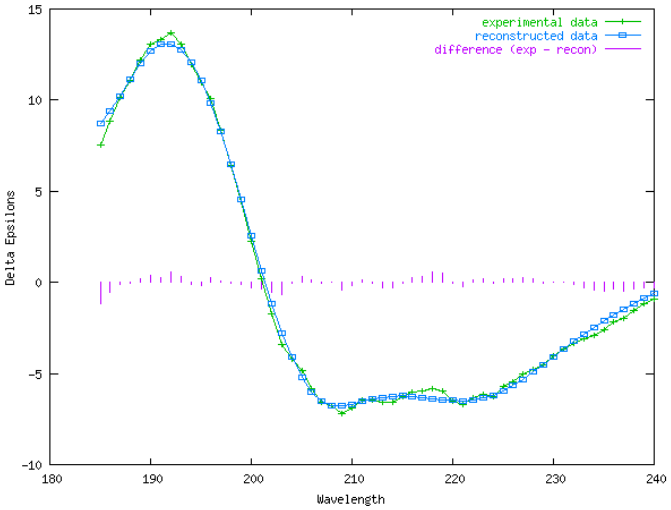


Figure S13. CD spectrum of cHSA-PEG(2000)$_{19}$-SE1$_5$ (**6**).

18. Enzymatic degradation of the hydrogel

DNase I (40 µL, 2.5 Units/µL in 1x reaction buffer*) was added to GFP loaded hydrogel (10 µL) in a PCR tube. The reaction solution was incubated at 37 ^{o}C. At desired time point, the solution was immediately cooled down in ice-cold water and 20uL of the solution was used to measure the fluorescence intensity (λ_{ex} = 460 nm and λ_{em} = 512 nm) using low volumn 384-well plate (Grenier Bio-one) on TECAN Infinite 1000 microplate reader. After measurement, the solution was immediately added back to the reaction and the enzymatic reaction was continued at 37 ^{o}C. The GFP concentration was calculated based on a calibration curve with GFP standard solutions at the same condition. The increasing of GFP concentration in the supernatant was plotted as shown in Figure 3c.

For trypsin digestion, trypsin (40 µL, 1 mg/mL in PBS buffer) was added to GFP loaded hydrogel (10 µL) in a PCR tube. The experiment was perform as same as for DNase I. It is known that GFP is highly resistant to trypsin digestion[3].

*1x DNase I reaction buffer contains 10mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 0.13mM $CaCl_2$.

19. Cell culture

A549 cells, a human alveolar basal epithelial carcinoma cell line, were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig). The primary foreskin fibroblast cells are obtained from Institute of human genetics, Ulm University, Germany. The research was carried out in complicance with the Helsinki Declaration, obtained written ethics

approval from the ethics committee (Ethikkommision Universität Ulm, A 185/09) and written informal consent from all participants. Both cells were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.1 mM non-essential amino acids at 37°C in a humidified 5% CO_2 incubator.

20. Preparation of hydrogel for 3D cell culture

A549 or fibroblast cells were trypsinated at 80% confluent, and prepared into 10000 cells/μL solution in completely supplemented DMEM medium. To prepare 50 μL 5% hydrogel with cells encapsulated inside, denatured HSA-SE1 solution (15%, 11.26 μL) was mixed with 5x TBE buffer (10 μL) and freshly prepared cell solution (17.46 μL), then the **DL1** linker solution (2 mM, 11.26 μL) was added and immediately mixed with carefully stirring. A transparent hydrogel with ~3500 cells/mm^3 was formed directly inside μ-Slide 8 well chamber (Ibidi, Martinsried, Germany). The fully supplimented DMEM medium was then added on top of the hydrogel and incubated at 37oC in a humidified 5% CO_2 incubator. Notably, FBS and DMEM medium do not contain sufficient nuclease or proteases that could degrade the hydrogel.

21. Live-cell imaging using laser scanning confocal microscope

After incubation of the 3D cell cultured hydrogel for 48 hrs, 1 μL of calcein-AM (1 mg/mL solution in DMSO) and 1 μL of propidium iodide (PI, 1 mg/mL solution in DMSO) was added to the incubation chamber and incubated for 5 mins before imaging. The hydrogel in μ-Slide 8 well chamber was directly imaged on LSM710 laser scanning confocal microscope (Zeiss, Germany) coupled to XL-LSM 710 S incubator and equipped with a 20× objective. Calcein was excited with 488nm Argon laser and PI was excited with 563 nm helium-neon gas laser respectively. Imaging was perform with z-stacking over 400 μm. The 3D graph was constructed using Zen 2010 software (Zeiss, Germany).

22. Cytotoxicity assay

A549 and Fibroblast cells were plated into a white 96 well half area microplate at a density of 5000 cells per well in 50μl complete DMEM and incubated overnight for attachment. The desired amount of **DL1**, HSA-SE1 conjugates and the hydrogel fragments after DNase I digestion (95^oC preheated for 10 min to inactive DNase I) were added to each well. All concentrations were prepared as quadruplicate. After further incubation with drugs for 24hrs, cell viability was tested by Celltiter-Glo™ (Promega) cell viability assay kit according to manufactory's instruction. Cells without treating by any drug were considered as blank.

References:

(1) Beaucage, S. L. & Iyer, R. P. Advances in the synthesis of oligonucleotides by the phosphoramidite approach. Tetrahedron 1992, 48, 2223-2311.

(2) Kuan, S. L.; Stöckle, B.; Reichenwallner, J.; Ng, D. Y. W.; Wu, Y.; Doroshenko, M.; Koynov, K.; Hinderberger, D.; Müllen, K.; Weil, T. Biomacromolecules 2013, 14, 367-376.

(3) Pesce, D.; Wu, Y.; Kolbe, A.; Weil, T.; Herrmann, A. Biomaterials 2013, 34, 4360-4367.

(4) Chiang, C.-F.; Okou, D. T.; Griffin, T. B.; Verret, C. R.; Williams, M. N. V. Archives of Biochemistry and Biophysics 2001, 394, 229-235.

(5) Fahrer J, Kuban J, Heine K, Rupps G, Kaiser E et al. Cell Microbiol 2010, 12, 233-247.