Electronic Supplementary Material

Three-dimensional printing of pure proteinaceous microstructures by femtosecond laser multi-photon crosslinking

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Laser properties

Table S1 Laser Properties: Gaussian beam profile and linear polarization remained constant for the laser alignment. Beam collimation and apertures were adjusted to achieve the final result of ca. 8 mm diameter at the backend aperture of the lens.

device	***Mikan (Amplitude Systemes)***	***D-1000-CE-3 (IMRA America)***	***DX210 (IMRA America)***	***DX210 (IMRA America)***	***<General>***
type	oscillator	fibre	fibre	fibre	either
pulsewidth [fs]	400	397	353	340	340-400
repetition rate	54 MHz	200.1 kHz	199.8	199.9 kHz	200 kHz or 54 MHz
central wavelength [nm]	1000-1100	1041.5	1049.6	1048.1	1000-1100

Noisy data exclusion rule for Raman data

A customized microscopic Raman spectrometer equipped with a 633 nm excitation He-Ne laser (Horiba, Japan), 100x objective lens, and spectrometer grating with 1800 grooves/mm was used and data were collected using LabSpec (Horiba).

It happened that erroneously the focus was not accurate and Raman data was obtained, which does not actually represent the sample. Furthermore, in rare occasions, laser interaction with the proteinaceous material caused visible damage and changes in the Raman spectrum. To exclude these cases as well as other possible errors of conduct, we developed a simple exclusion rule tailored to our data set: **After normalization** (to the 997 1/cm Raman shift for protein related data or to the Raman shift with maximal peak for the other data)**, the average over all Raman shifts in one spectrum shall be smaller than 0.4.**

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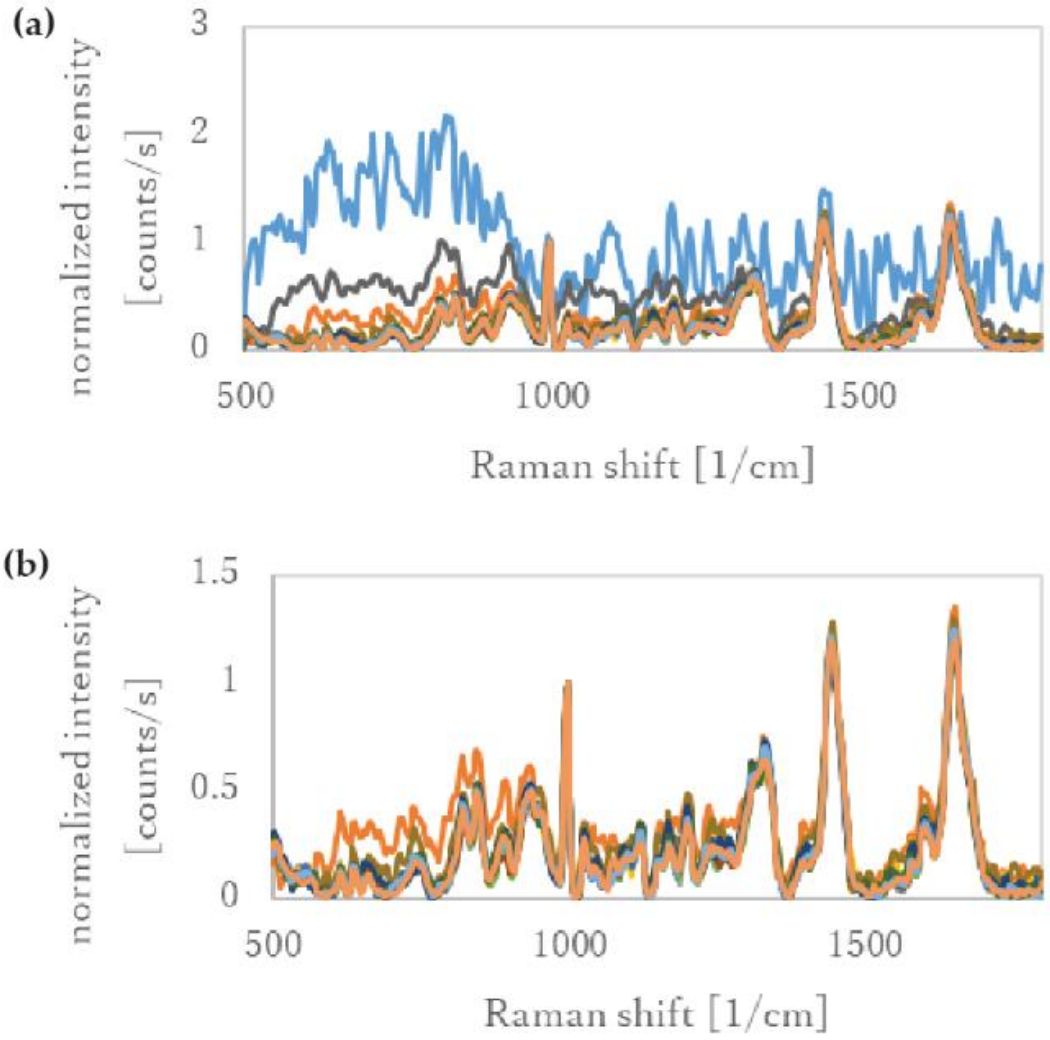

Figure S1 Noise Removal Process

For Figure 3, 36 physically independent samples were measured in total 170 measurements. Of these 170 measurements, 33 measurements (20%) were rejected because after normalization the average of values was greater than 0.4. (a) One data set of BSA film is shown. The blue data scored 0.97, the brown data scored 0.48. (b) After removal, the graph looks as shown.

SEM images demonstrating acid-catalyzed hydrolysis

In the manuscript, our analysis focusses on the degradation observed by top-down microscopy. Here, we show SEM images of key aspects of the procedure. However, because the hydrogel-like structures are soft and mechanically not very robust, structures can be seen collapsed or sunken into themselves. Air-drying preceded all hydrolysis experiments in order to avoid the introduction of unknown liquid

amounts into hydrochloric (HCl) concentration, and to avoid the use of SEM fixation protocols that may alter the proteinaceous microstructures chemically. For conductivity, all samples were coated with carbon.

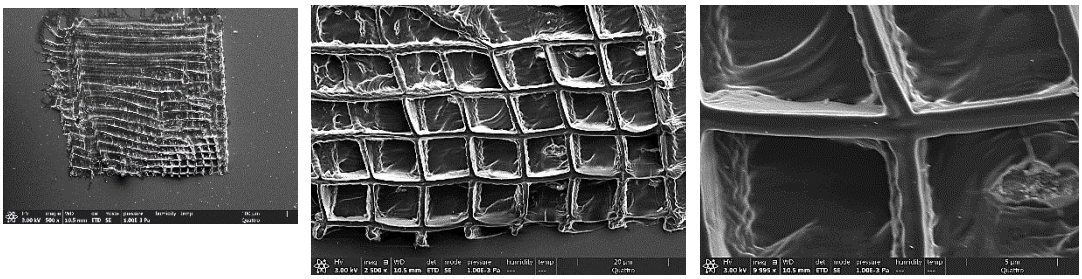

Figure S2 BSA microstructure, air-dried, no treatment.

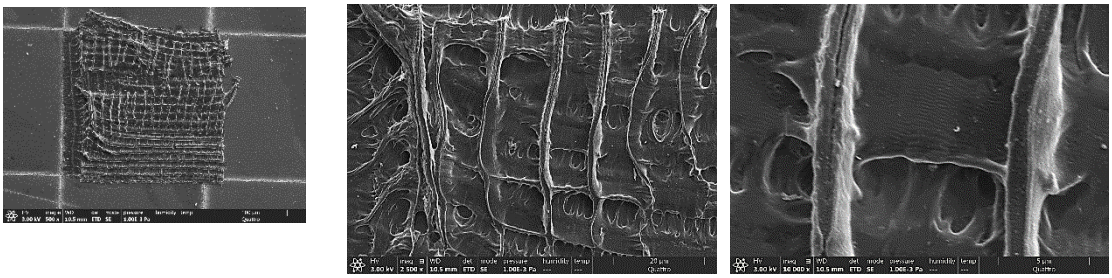

Figure S3 BSA microstructure, air-dried, 110^{O}C in water for >40 min, and air-dried.

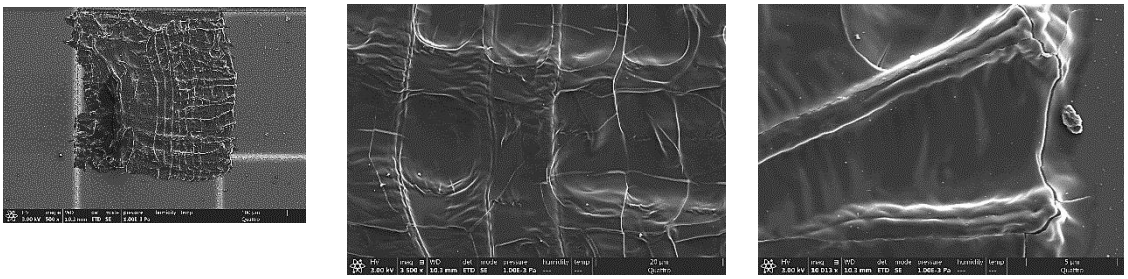

Figure S4 BSA microstructure, air-dried, room temperature in HCl for 40 min, washed in water to rinse the HCl, and air-dried.

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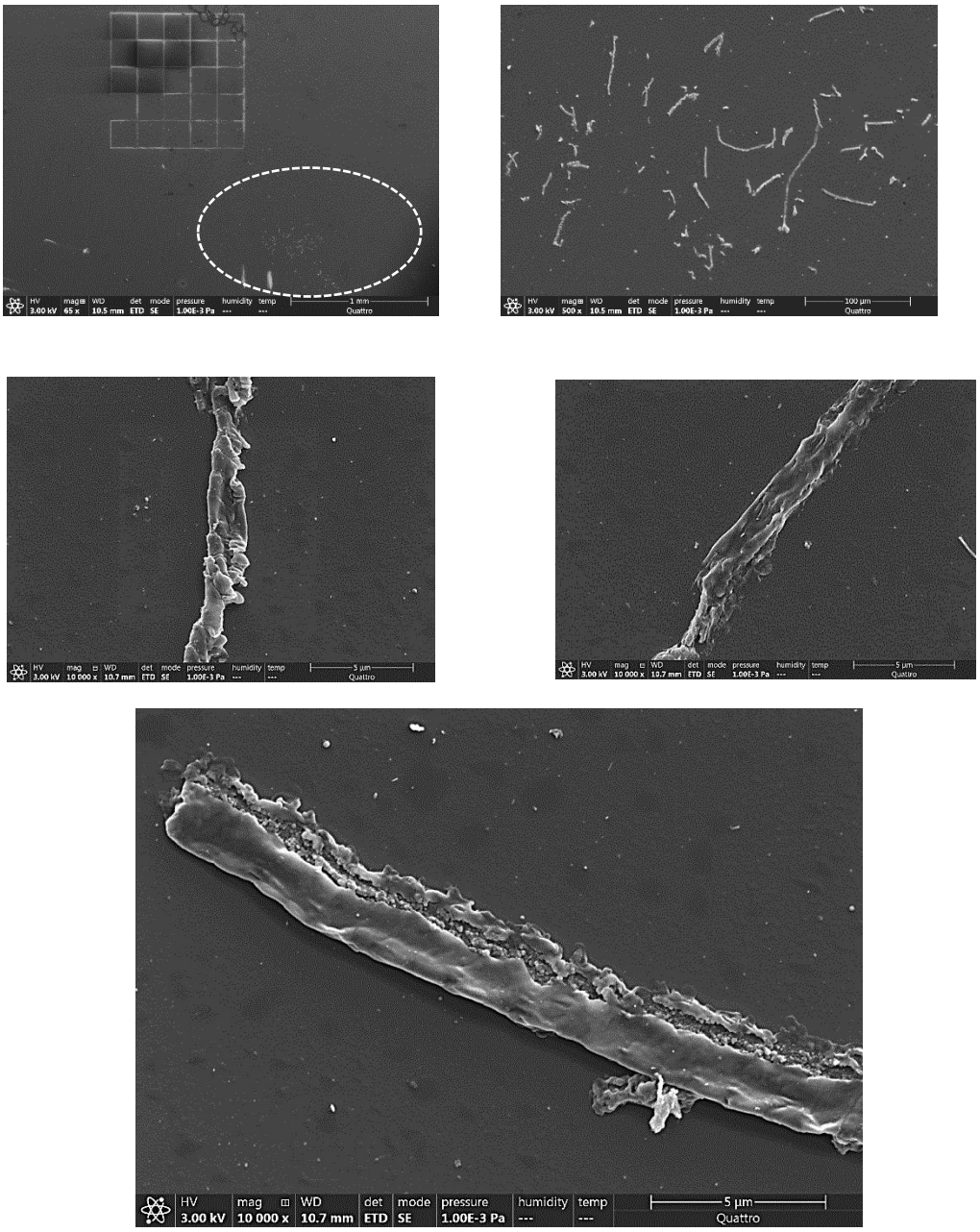

Figure S5 BSA microstructure, air-dried, 110°C in HCl for 10 min

These structures were washed in water to rinse the HCl, and air-dried. The structure was notable by bare eye and disintegrated when entered into water for rinsing. With careful handling and luck, the pieces could be traced from the original structure location on the ablation grid to the location at the time of drying, using optical microscopy.

Not only did the hydrolyzed structure disintegrate into pieces when transferred to water for washing, but also the **remaining pieces seem to be less voluminous** in comparison with the other conditions shown above.

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MM-scale sample for ATR FT-IR spectroscopy

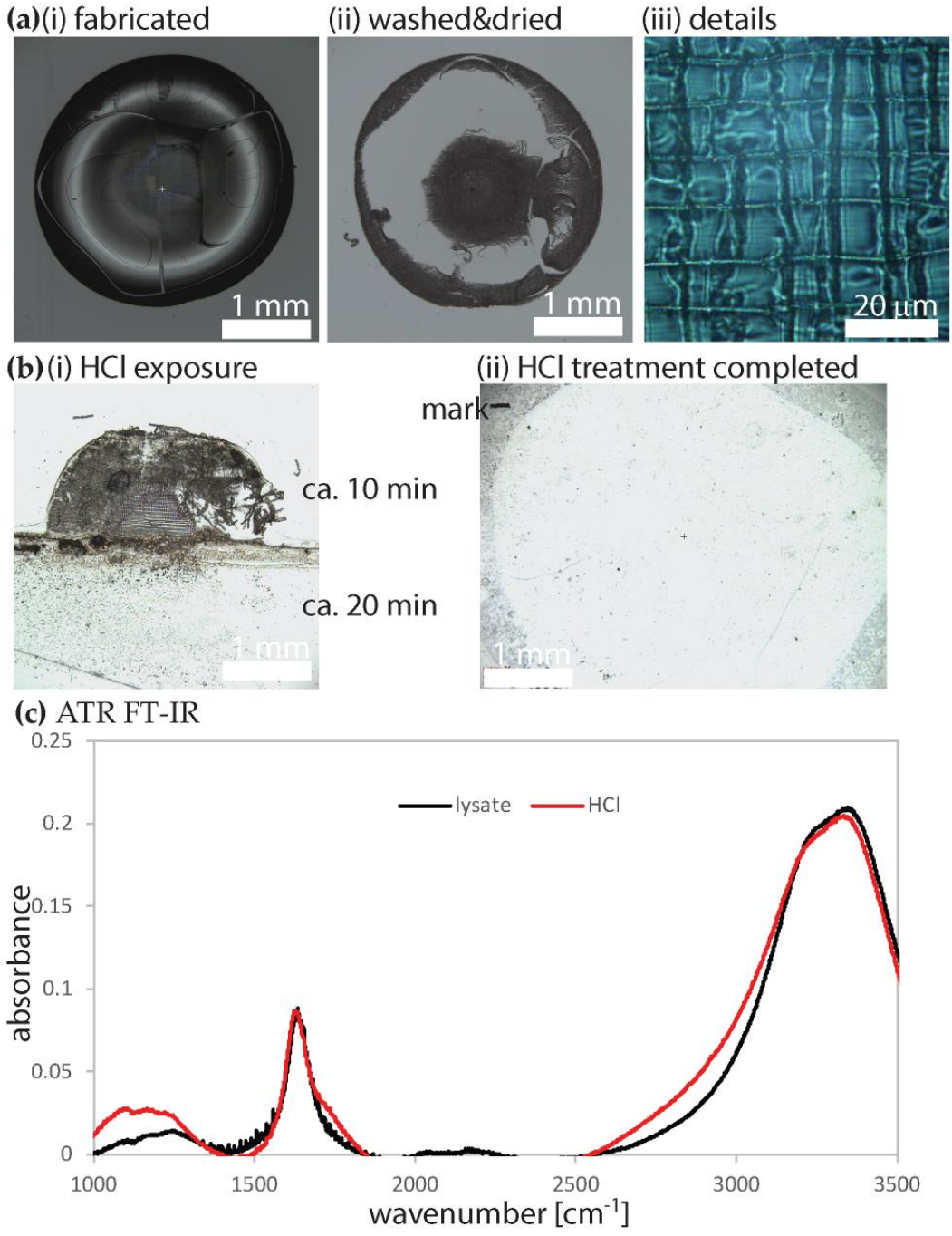

Figure S6 Serum albumin sample for ATR FT-IR spectroscopy

Films of 200 mg/mL protein solutions were spin-coated. Structures were fabricated with 20x N.A.= 0.46 air lens, 10 mW laser power, 100 µm/s scanning speed and a 200 kHz fiber laser with pulse width 397 fs. (a) Optical microscopic images show (i) the film of BSA with inscribed structure of crossing lines, (ii) rinsed and air-dried proteinaceous structure, and (iii) close-up of the central area showing the resulting crossing line pattern. (b) Optical microscopic images show proteinaceous film exposed to HCl. (i) A vertical exposure allowed to study two conditions, ca. 10 min and ca. 20 min. (ii) A completed HCl treatment leaves no trace of microstructures. (c) By ATR FT-IR spectroscopy, no relevant difference between lysate and 6 M HCl is observed.

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Expected feature sizes

Beam quality $M^2 = 1.3$, Numerical aperture N.A.=1.35, wavelength $\lambda = 525$nm

Radius of Gaussian beam: $w_0 = \frac{M^2 0.61\lambda}{N.A.}$; Diameter: $d = 2w_0 = 0.62\ \mu m$;

(Rayleigh length: $z_R = \frac{\pi w_0^2}{\lambda}$; Height: $h = 2z_R$; but diffractive effects are neglected.)

Diameter with two-Photon (2P) absorption processes: $d^{2P} = \frac{d}{\sqrt{2}} \sim 0.7d = 0.434\ \mu m$

Refractive index n for protein solution, $n_{ps} = \frac{Conc}{133}\left(1.58 + \left(\frac{133}{Conc} - 1\right)n_s\right) = 1.425$

considering the concentration of protein $Conc = 20\ grams/100mL$ and refractive index of solvent, $n_s = 1.398$, (ref. 31);

Diffractive effects considered, confocal length: $h = \frac{4n_{ps}\lambda}{N.A.^2} = 1.6\ \mu m$; (ref. 32),

Height with two-photon processes and diffractive effects: $h^{2P} = \frac{h}{\sqrt{2}} \sim 0.7h = 1.16\ \mu m$

FAD Raman data

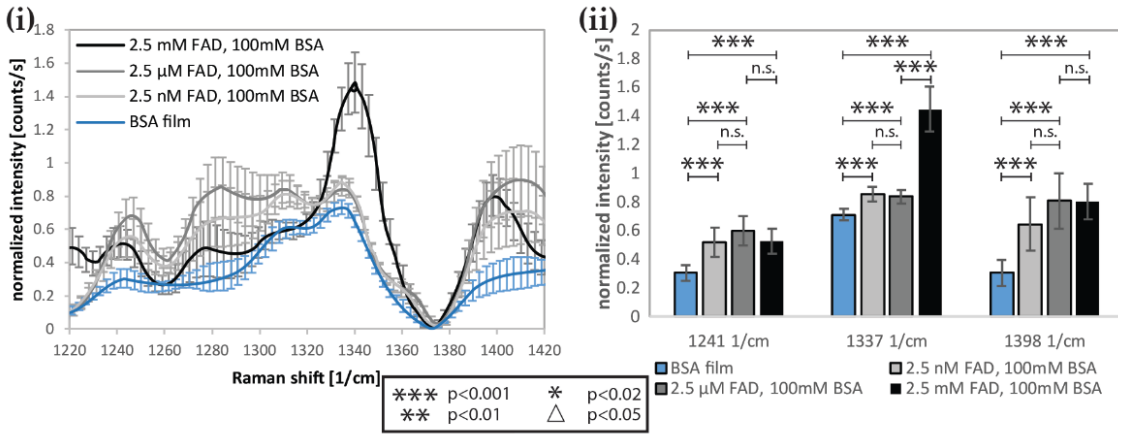

Figure S7 BSA with FAD film and pure BSA film
Raman data of FAD mixed with BSA at different ratios in dried drop-casts. (i) Excerpt of 1220 to 1420 cm-1 Raman shifts (ii) Bar graph shows significant differences between pure BSA film and FAD mixtures. Error bars show standard deviation. Two-tailed t-tests were conducted with the confidence level of 95%.

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Anti-BSA antibody staining of BSA microstructure

A microstructure of BSA was fabricated with 20x N.A.= 0.46 air lens, 3 mW laser power, 5 μm/s scanning speed and a 199.9 kHz fiber laser with pulse width 340 fs. After air-drying and several weeks of storage, the structure was imaged at 50x magnification with an Olympus microscope BX 51, 1 s data acquisition for white light source excitation filtered to be 450-480 nm and emission was filtered 520-800 nm.

First, we performed 1 hr incubation at room temperature with 28 μg/mL anti-BSA antibody produced in rabbit (SAB4301142, Sigma Aldrich). After rinsing with purified water, we continued with 1 hr incubation at room temperature with 40 μg/mL goat produced anti-Rabbit IgG (H+L) Secondary Antibody, Alexa Fluor® 488 conjugate (A-11008, Invitrogen/ThermoFischer)

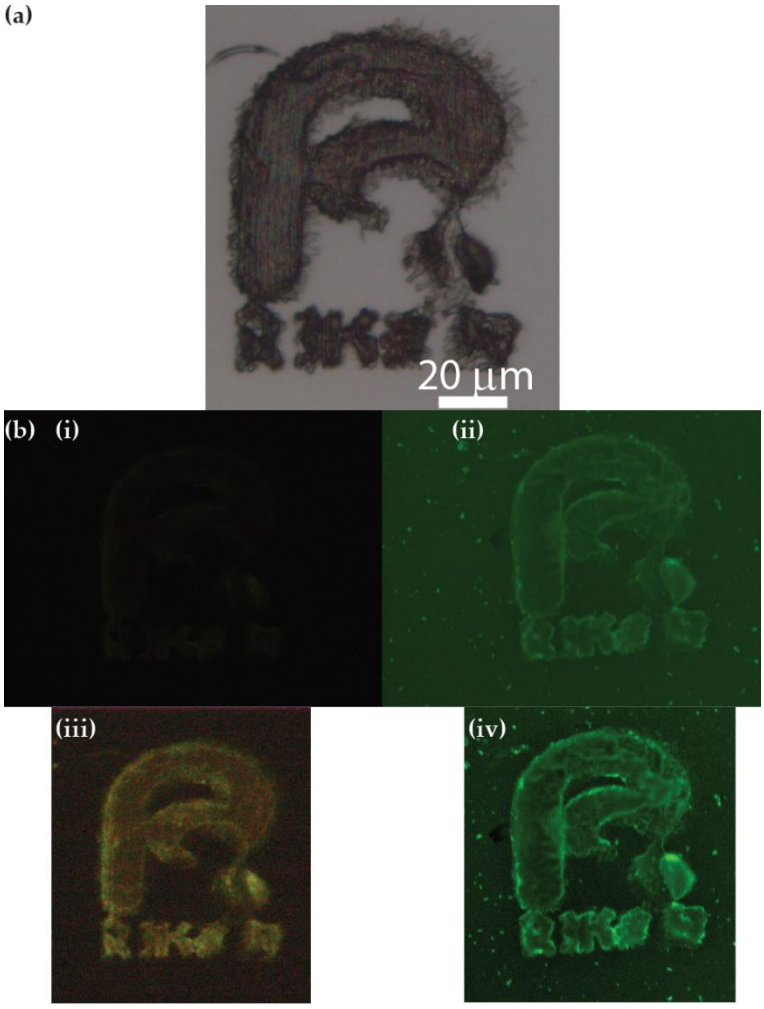

Figure S8 anti-BSA staining – binding function confirmation

RIKEN logo made from BSA was exposed to anti-BSA and secondary antibody. (a) Bright field image. (b) Raw images were obtained with 1 s acquisition for (i) before (dry) and (ii) after staining (liquid). (iii) Extreme enhancement of brightness and contrast reveals the structure before staining due to autofluorescence. (iv) Mild enhancement increases the features of RIKEN logo after successful staining.