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Article

One-step photochemical crosslinking of native proteins is feasible in tyrosine-rich bovine serum albumin

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SUMMARY

Hydrogels made by crosslinking biomolecules have wide-ranging applications, including pharmaceuticals and tissue engineering. Some research groups have demonstrated crosslinking polycarbonates or oligopeptides by first attaching a tyrosine crosslinker to the backbones and then initiating crosslinking among linkers. Herein, we explored an alternative approach to make hydrogels from native proteins without pre-modification. We hypothesized that it is feasible to photochemically crosslink unmodified proteins provided that the protein is naturally abundant in tyrosine groups. We chose bovine serum albumin (BSA, a protein containing 18 tyrosine residues) as the native protein and methylene blue (MB) as the photosensitizer. To test our hypothesis, we photo-illuminated a solution of BSA and MB and observed whether a hydrogel formed. After illumination, the solution became a gel-like film whereas the solution not subject to illumination remained fluidic, indicating that photochemically crosslinking unmodified proteins is feasible. Using a microscope objective lens to focus the laser beam and a stage to move the sample, we further demonstrated photo-printing finely structured hydrogels. To illustrate a potential application of the hydrogels, we showed that the structured hydrogels can incorporate a model drug (fluorescein), and can be transferred and adhered to a model skin (pig skin), acting like a liquid bandage. In conclusion, we verified the feasibility of making hydrogels from a native protein naturally rich in tyrosine without the extra step of introducing crosslinkers to the protein backbone and demonstrated photo-printing fine structures of protein hydrogels. More work will be necessary to explore novel applications of protein hydrogels.

INTRODUCTION

Hydrogels made of an assembly of protein molecules have high biocompatibility and water-absorbing capability and can be used to develop various applications including drug carriers for controlled release, wound dressing, biomedical implants, regenerative medicine, and tissue engineering (1). Protein molecules can self-assemble into hydrogels through either noncovalent or covalent interactions (2, 3). Since noncovalent interactions, which include electrostatic attraction, hydrogen bonding, π-π stacking, and hydrophobic interaction, tend to be weak, protein hydrogels made through noncovalent bonding may fall short due to mechanical instability. On the contrary, protein hydrogels made through strong covalent bonding have the potential to become a biomaterial with improved strength, elasticity, or stiffness.

Interestingly, the formation of some naturally occurring biomaterials, such as the fibroblast-derived extracellular matrix (ECM) or the rubber-like resilin protein, also involves covalent bonding (4). Specifically, the tyrosine moiety of proteins acts as a "crosslinker", and covalent bonding between proteins forms through oxidative crosslinking between the tyrosine moieties (4). To initiate oxidative crosslinking between tyrosine moieties, an enzyme first catalyzes the formation of tyrosine free radicals, and then the tyrosine free radical combines with a proximal tyrosine radical to form dityrosine (**Figure 1**).

Following this line of reasoning, one may crosslink polycarbonates or oligopeptides by introducing crosslinkers to the backbone of these species. For instance, one research group attached tyramine molecules to alginates through the formation of an amide bond between the amine group of tyramines and the hydroxyl group of alginates, and then initiated chemical crosslinking between tyramine-modified alginates with a strong oxidizing agent (5). To crosslink artificial oligopeptides, another group synthesized tyrosine-rich oligopeptides and assembled these oligopeptides into diverse nanostructures through photochemical crosslinking (6).

Inspired by mother nature and the aforementioned pioneering works, we propose an alternative biomimetic approach to create hydrogels from native proteins without pre-modification and without introducing crosslinkers to the backbone of proteins. As the tyrosine radical is highly reactive and hence has a short lifetime, tyrosine crosslinking is effective only if the distance between tyrosine moieties is

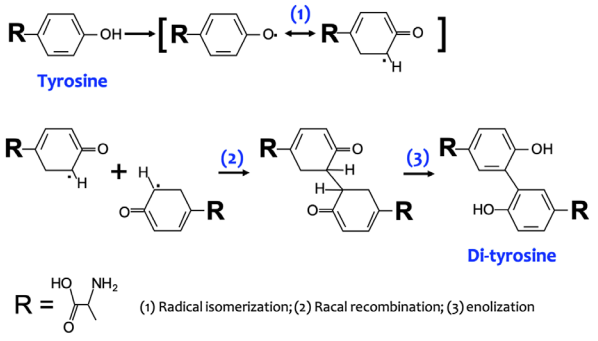

Figure 1: Schematic showing how two tyrosine groups crosslink, forming dityrosine through a radical reaction. To initiate oxidative crosslinking between tyrosine moieties, an enzyme first catalyzes the formation of tyrosine free radicals, and then the tyrosine free radical combines with a proximal tyrosine radical to form dityrosine.

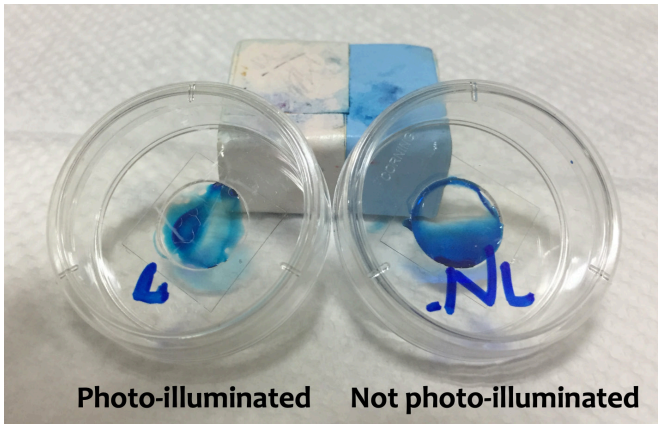

Figure 2: Verification of photochemical crosslinking of BSA. Photo showing two Petri dishes that contain a solution of BSA and MB. The solution subject to photo-illumination (left) became a gel-like film; in contrast, the solution for the control that was not subject to photo-illumination (right) remained fluidic. The result provides strong evidence that photochemically crosslinking BSA is possible.

small. Accordingly, we hypothesized that crosslinking native proteins may be feasible provided that the protein is abundant in tyrosine groups. Similar to the photochemical crosslinking of artificial tyrosine-rich oligopeptides, we sought to create tyrosine free radicals through photochemical reactions.

In this work, we chose bovine serum albumin (BSA), a common mammalian protein that contains 18 tyrosine residues among its 583 amino acids (7), as the native protein. BSA has a high level of tyrosine residues and hence is an ideal protein for us to explore our hypothesis. The photosensitizer is a molecule that can facilitate a chemical change in another molecule through a photochemical process. To assist in photochemical crosslinking of BSA, we chose methylene blue (MB) as the photosensitizer in this study because of its low cost and high biosafety to humans (8). To test our hypothesis, we prepared a solution comprising BSA and MB and photo-illuminated the solution with an LED. We then observed whether a hydrogel film was formed after photo-illumination. Our result showed that the protein solution became a gel-like film after photo-illumination, indicating that crosslinking of unmodified proteins is feasible. Using a tightly focused laser beam, we further demonstrated photo-printing fine structures of protein hydrogels. Our approach may be extendable to other proteins that have a high level of tyrosine residues. The capability to "print" hydrogels with laser illumination further opens up the possibility to fabricate hydrogels with versatile functionalities such as scaffolds for tissue engineering.

RESULTS

Verification of photochemical crosslinking of BSA

The objective of this work was to verify if one can produce hydrogels from unmodified tyrosine-rich proteins through photochemical crosslinking. To test this hypothesis, we distributed a solution comprising BSA and MB to two Petri dishes and then photo-illuminated one petri dish with a red LED. The other Petri dish maintained in the dark served as the control. As shown in **Figure 2**, the solution subject to photo-illumination became a gel-like film; in contrast, the control solution that was not subject to photo-illumination remained fluidic. These results provide strong evidence that the photochemical crosslinking of BSA is possible.

Creation of finely structured protein hydrogels with laser-assisted photo-illumination

Having demonstrated the feasibility to create BSA protein hydrogels through photochemical crosslinking by illuminating the protein solution with an LED, we next sought to verify if one can create finely structured protein hydrogels with unmodified BSA. Specifically, we placed the Petri dish on a microscope and illuminated the solution with a tightly focused light beam from a near-infrared pulsed laser.

Singlet oxygen has been known to mediate the photosensitized crosslinking of proteins, and the lifetime of singlet oxygen is longer in heavy water (D_2O) than that in normal water (H_2O) (9). Accordingly, we hypothesized that heavy water might be a preferable solvent relative to normal water. We first compared hydrogels made from BSA prepared in either normal water or heavy water. As shown in **Figure 3a**, laser-illuminating a BSA/D_2O solution resulted in finely structured protein hydrogels. Formation of hydrogels dots succeeded even for the shortest exposure time (10 s) tested in this experiment and gradually became sharper and more focused as the exposure time increased (10 s, 15 s, 30 s, 45 s, and 60 s). In contrast, even though the general trend was similar, the formation of hydrogels was unsuccessful for the BSA/H_2O solution unless the exposure time was increased to 30 s or longer. This result leads to the conclusion that heavy water is a preferred solvent because photo-crosslinking BSA proteins in D_2O yielded better results and required smaller exposure time. This can be explained by the fact that singlet oxygen has a longer lifetime in D_2O than H_2O and therefore more singlet oxygen molecules are available for facilitating photochemical crosslinking. Besides, while our result showed that an exposure time of 10 s was sufficient to generate hydrogel dots when using D_2O as the solvent, we chose an exposure time of 45 s in the next experiment.

We next investigated how the laser focus (-2 µm, 0 µm, +2 µm, +4 µm, and +6 µm depth relative to the surface of the glass substrate) affected the formation of hydrogels. Based on the preceding result, we prepared the solution in heavy water and set the exposure time to 45 s. As shown in **Figure 3b**, the focal point had a strong effect on the formation of hydrogels. In particular, the fabrication failed if the focal points were set at -2 µm and 0 µm relative to the surface of the substrate. On the other hand, raising the focal point to 6

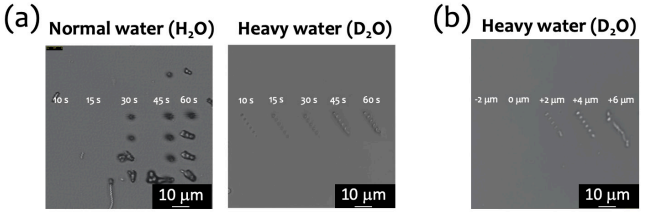

Figure 3: Optimization of experimental conditions to produce protein hydrogels with photochemical crosslinking. (a) Comparison of the results produced with solutions prepared in different solvents (left panel: normal water (H_2O); right panel: heavy water (D_2O)) and with different exposure times of photo-illumination (10 s, 15 s, 30 s, 45 s, and 60 s) with a fixed focal depth of +4 µm. (b) Comparison of the results produced by adjusting different focal depths (-2 µm, 0 µm, +2 µm, +4 µm, and +6 µm relative to the upper surface of the glass) using a solution prepared in D_2O and an illumination time of 45 s.

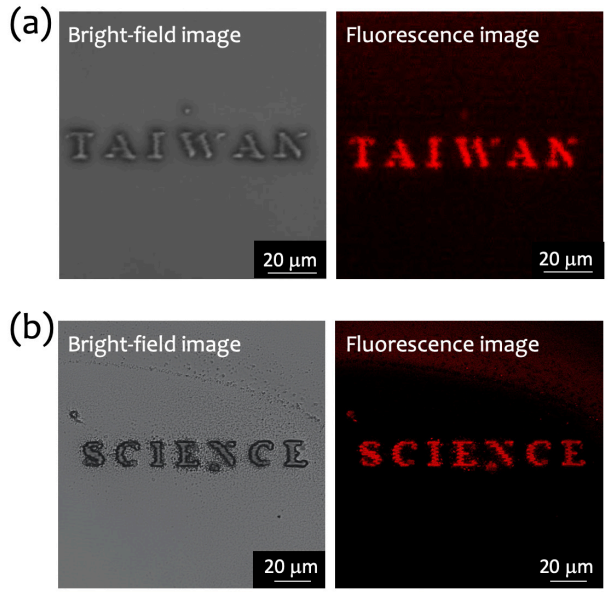

Figure 4: Demonstration of the fabrication of text patterns with protein hydrogels. (a) Bright-field (grey) and fluorescence (red) images of a text pattern of "TAIWAN" produced with protein hydrogels. (b) Bright-field (grey) and fluorescence (red) images of a text pattern of "SCIENCE" produced with protein hydrogels. The red fluorescence was produced from residual MB in the hydrogel with excitation using a 633 nm laser.

mm above the surface did not produce patterns well-adhered to the surface, and the shape of the patterned hydrogel dots was irregular and inconsistent when the focal point was set at 2 μm above the surface. Setting the focal point 4 μm above the surface produced the optimal result. The hydrogel dots were well adhered to the surface and their size and shape were very consistent. Of note, hydrogel formed only near the laser focus where the laser intensity had the maximum, which is consistent with the hypothesis that hydrogel formed through photochemical crosslinking. Moreover, our result shows that the size of the hydrogel dots scaled with the exposure time. This result indicates that controlling the size of the fabricated hydrogel patterns by adjusting the exposure time may be promising.

Fabricating protein hydrogels with designed patterns

We next sought to explore the possibility of fabricating hydrogels of user-designed patterns on a glass surface. To achieve this, we placed the Petri dish on a computer-controlled translation stage that was mounted on an optical microscope.

We created two text patterns of "TAIWAN" and "SCIENCE" with hydrogels by photo-crosslinking BSA on a glass substrate. As MB is a fluorescent molecule and some MB residues presumably remained in the hydrogel, fluorescence imaging should allow us to inspect the quality of the text patterns. Accordingly, we set the excitation wavelength (633 nm) and the spectral window of detection to match the absorption and the fluorescence spectra of MB. We displayed the bright-field and fluorescence images of the two text patterns of BSA hydrogels in **Figure 4**. While some minor defects existed, the results demonstrated that the patterned hydrogel nicely reproduced the texts in the original patterns.

Fabricating patterned protein hydrogels that can incorporate drugs and transfer to the skin

A potential application of this research is to develop a novel "band-aid" made with protein hydrogels. Ideally, we anticipate the bandage should have the following features. First, the bandage should be made of protein hydrogels based on designed patterns. Second, the patterned hydrogel should incorporate drugs for medication. Third, the patterned hydrogel can be transferred to the skin and adhere to the skin. To achieve the above goals, we designed the following three experiments.

First, we demonstrated the capability of creating arrays of dotted hydrogels of various densities. Similar to the preceding experiments, we created arrays of hydrogels with laser illumination. We showed the bright-field and fluorescence images of the hydrogel arrays in **Figure 5**. Images of a 5-by-5 array of dotted hydrogels with both lateral (Δx) and vertical (Δy) distances set at 10 μm, a 5-by-5 array with the Δx and Δy distances set at 20 μm and 10 μm, and a 5-by-5 array with both the Δx and Δy distances set at 30 μm are displayed in **Figures 5a**, **5b**, and **5c**, respectively. The above results clearly demonstrate that we were able to fabricate dotted arrays of hydrogels with user-controlled density and patterns with high accuracy.

Second, we sought to explore the capability of incorporating drugs into patterned protein hydrogels for medication. We utilized fluorescein molecules as a model "drug" to mimic medication. As fluorescein is a fluorescent molecule, we were

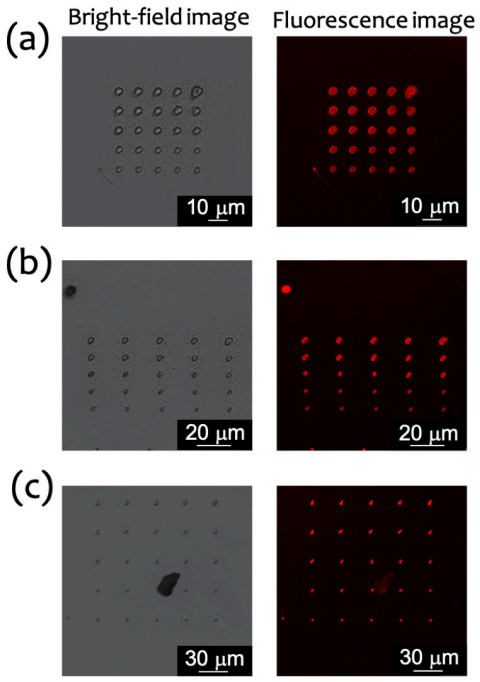

Figure 5: Demonstration of the fabrication of arrays of patterned dots of protein hydrogels arranged at different distances. (a) Bright-field (grey) and fluorescence (red) images of an array of patterned dots of protein hydrogels (distance between dots: Δx = 10 μm and Δy = 10 μm). (b) Bright-field (grey) and fluorescence (red) images of an array of patterned dots of protein hydrogels (Δx = 20 μm and Δy = 10 μm). (c) Bright-field (grey) and fluorescence (red) images of an array of patterned dots of protein hydrogels (Δx = 30 μm and Δy = 30 μm) The red fluorescence was produced from residual MB in the hydrogel with excitation using a 633 nm laser.

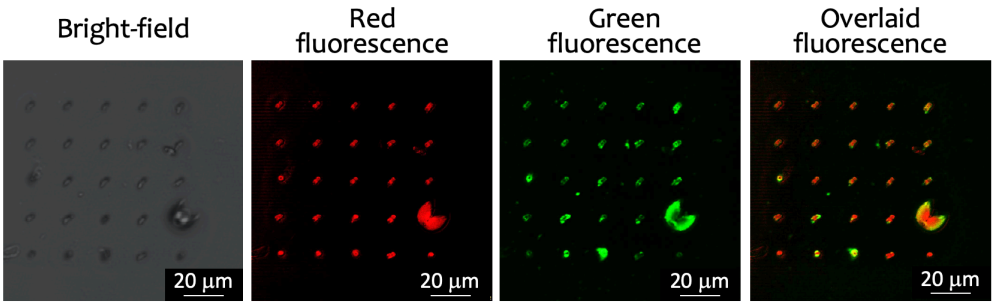

Figure 6: Demonstration of the incorporation of a model drug (fluorescein) into protein hydrogels. Bright-field (grey), red fluorescence, green fluorescence, and overlaid fluorescence (red and green) images of an array of patterned dots of protein hydrogels (distance between dots: Δx = 20 μm and Δy = 20 μm) after incorporating fluorescein that acted as a model drug for the sake of demonstration in this study. The red fluorescence was produced from residual MB in the hydrogel with excitation using a 633 nm laser, whereas the green fluorescence was produced from the fluorescein molecules incorporated in the hydrogel with excitation using a 488 nm laser.

able to verify whether the model "drug" was incorporated into or adsorbed onto patterned hydrogels with fluorescence imaging. To avoid possible photodamage of drugs during photo-crosslinking, we incorporated drugs after the protein hydrogels form. To test this idea, we created a 5-by-5 array of dotted protein hydrogels on a glass substrate, soaked the substrate in a solution of fluorescein, and then took optical images after rinsing the substrate with deionized (DI) water. **Figure 6** displays the bright-field, red fluorescence, green fluorescence, and overlaid fluorescence images acquired from the resulting 5-by-5 array of dotted protein hydrogels after rinsing. Specifically, the red fluorescence was produced from the emission of residual MB in the protein hydrogel, whereas the green fluorescence was produced from the emission of the incorporated fluorescein molecules. The green fluorescence image and the overlaid red and green image provide strong evidence that the model "drug" was successfully incorporated into the patterned protein hydrogels.

Finally, we sought to explore the possibility of producing user-designed patterns of hydrogels that are easily transferable to skin. Specifically, we utilized porcine skin to mimic human skin. We fabricated a 3-by-3 array of BSA hydrogel dots on a thin, transparent plastic sheet using the same method as the fabrication of hydrogel dots on a glass substrate. The photo displayed in **Figure 7a** shows the 3-by-3 array of BSA hydrogel dots that was fabricated on the plastic sheet. We then transferred the array of hydrogel dots to a piece of porcine skin by gently pressing the plastic sheet onto the skin. The photo displayed in **Figure 7b** demonstrates that the transfer of protein hydrogel dots onto the porcine skin was successful, and these hydrogel dots adhered well to the skin after transferring.

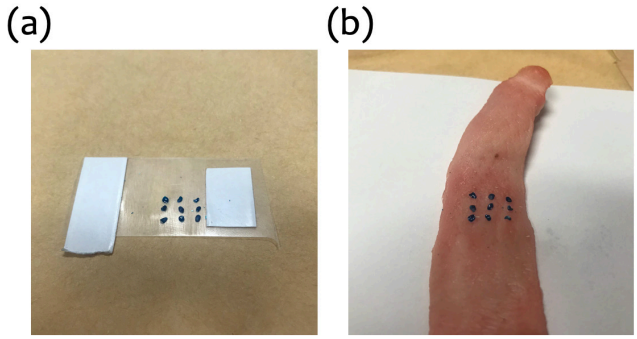

Figure 7. Demonstration of transferring patterned dots of protein hydrogels to porcine skin. (a) Photo of an array of patterned dots of protein hydrogels produced on a thin and transparent plastic sheet. (b) Photo of a piece of porcine skin showing that the array patterned dots of protein hydrogels was transferred and adhered to the skin.

DISCUSSION

The major objective of this study was to verify the hypothesis of photo-crosslinking native tyrosine-rich proteins such as BSA. We found that it is feasible to photochemically crosslink unmodified BSA (**Figure 2**). According to the literature, photosensitized crosslinking of proteins can be mediated by singlet oxygen, and the lifetime of singlet oxygen is longer in heavy water than that in normal water (9). Consistent with the aforementioned, our results show that heavy water is a preferable solvent compared to normal water for preparing solutions of BSA for protein hydrogel fabrication through photosensitized crosslinking (**Figure 3**). These results also suggest that singlet oxygen plays an important role in BSA photosensitized crosslinking.

We also explored the possibility of creating finely structured protein hydrogels. Our results show that the size of the fabricated hydrogel dots strongly correlates with the duration of photo-illumination (**Figure 3**), indicating the unique possibility to control the hydrogel size with the exposure time. Facilitated with a computer-controlled translation stage, we moved the sample relative to the focus of the laser and controlled the exposure time at each position. Besides, we demonstrated the possibility of fabricating hydrogels in user-designed text patterns (**Figure 4**) and arrays of hydrogel dots with various densities (**Figure 5**). Furthermore, we showed that the dotted protein hydrogels can incorporate fluorescein molecules, which acted as a model drug in this work (**Figure 6**), and demonstrated a method to transfer designed patterns of protein hydrogels onto the skin (**Figure 7**). Considering the above results together, we have shown that BSA protein hydrogels have the potential to become an alternative to adhesive bandages. Importantly, through the fabrication of hydrogel dots of various densities and sizes, it is possible to control the drug quantity incorporated in a bandage. Although we have only demonstrated transferring hydrogels onto a plain sheet of porcine skin, the hydrogels can potentially be fabricated on bendable or molded substrates. In other words, the bandage can be custom-made to fit wounds in different regions of the human body where conventional band-aids are otherwise not suitable.

While our study has demonstrated the potential to develop protein hydrogels as an alternative biomaterial for novel bandages, we recognize that there are limitations and that some questions remain unanswered. As we fabricated patterns by moving the substrates relative to the laser focal point, the procedure of creating hydrogel patterns is slow and the yield is suboptimal. Nevertheless, we suggest that one may create a "mask" of desired patterns and illuminate the mask with higher powered light so that mass production may be possible. Additionally, while our result of incorporating a model drug (fluorescein) into the protein hydrogels is encouraging, it is unclear whether these molecules can release from the protein hydrogels. Furthermore, we used an excised porcine skin to model human skin, but we are not sure whether excised skin (i.e. dead tissues) has the same properties as the skin in living animals. Moreover, we utilized heavy water, MB, and BSA, a protein from animals, to fabricate hydrogels. Their relatively high cost and uncertain biocompatibility may become a hurdle preventing broad application as a biomaterial for adhesive bandages. In addition, we were unfortunately unable to test whether the drug is incorporated within the hydrogel or just on the surface due to the relatively small size of the hydrogel. As a result, more work and rigorous examinations are required on the toxicity and biosafety of hydrogels to apply the protein hydrogels as a biomaterial for bandages or beauty products. Alternatively, it is also valuable to find low cost and high biocompatibility components to substitute the animal protein, photosensitizer, or heavy water used in this study.

In conclusion, we verified that it is possible to create hydrogels from native proteins without the extra step of introducing crosslinkers to the backbone of proteins, and we demonstrated the photo-printing of fine structures of protein hydrogels. To explore the biomedical application of protein hydrogels, more work is necessary.

MATERIALS AND METHODS

Preparation of the solution for photochemical cross-linking of bovine serum albumin (BSA)

We prepared a stock solution of methylene blue (MB; Sigma-Aldrich) by dissolving 0.037 g MB powder in 20 mL of H_2O (or D_2O; Sigma-Aldrich) to a final concentration of 6.5 mM. Next, we pipetted 1.2 mL of the MB solution to an Eppendorf tube, placed 0.4 g bovine serum albumin (BSA; Sigma-Aldrich) into the Eppendorf tube to yield a final concentration of BSA of 6.1 mM, and sonicated the Eppendorf tube to ensure the solutes dissolved completely.

Formation of protein hydrogel with photochemical crosslinking of BSA

The photosensitizer (MB) has a strong absorption band around 660 nm (10). To induce photochemical crosslinking of BSA, a red LED or a near-infrared pulsed laser (ƛ = 1064 nm; picoTRAIN, High Q laser) was utilized to photoexcite MB through single-photon or two-photon absorption, respectively. Before experiments, we pipetted 200 µL of the MB and BSA solution to a glass-bottomed Petri dish. To induce photo-crosslinking with the LED, we placed the dish under the illumination of the LED. To induce photo-crosslinking with the laser, we placed the dish onto an inverted optical microscope (IX 71, Olympus). To optimize the experimental conditions for producing BSA hydrogels, we compared results produced from solutions prepared in different solvents (H_2O or D_2O), different exposure times (10 s, 15 s, 30 s, 45 s, and 60 s), and different focal depths of the laser beam (-2 µm, 0 µm, +2 µm, +4 µm, and +6 µm relative to the glass surface).

Fabrication of designed patterns of protein hydrogels

To produce a pattern of protein hydrogels, we employed a computer-controlled translation stage (P-563, Physik Instrument) to move the sample relative to the laser focus according to a user-designed pattern. The computer code (written in LabView) read a pattern that had been created with commercial software (such as MS PAINT), and then controlled the trajectory and speed of the translation stage according to the user-inputted pattern and settings. In this study, we tested two types of hydrogel patterns: an array of hemispherical dots, and text patterns made of straight lines. To fabricate an array of hydrogel dots, the exposure time at each designated position was 60 s. To fabricate the text, the translational stage was moved at a speed of 0.1 µm/s. The focal depth was fixed at +4 µm.

Incorporation of model drugs to protein hydrogels

To explore the possibility that photo-crosslinking hydrogels can incorporate drugs for future medical applications, we chose fluorescein molecules as a model drug for this study. We first created an array of hemispherical BSA hydrogel dots on a glass-bottomed Petri dish, and then immersed the hydrogel array in a fluorescein solution (1 µM) for 120 s. After rinsing the array with DI water several times, we inspected the sample with fluorescence imaging.

Confocal imaging of patterned hydrogels

We inspected the quality of patterned photo-crosslinking hydrogels with bright-field and confocal fluorescence images using a laser scanning confocal microscope (SP5, Leica). Depending on the fluorophores, a 488 nm laser or a 632.8 nm laser was employed for photoexcitation of fluorescein (11) or methylene blue (10), respectively.

Transferring patterned hydrogels to excised porcine skin

To explore the possibility that the patterned hydrogels can be transferred to the skin for future medical applications, we first created a hemispherical BSA hydrogel dots array on a thin and transparent plastic sheet. To mimic transferring patterned hydrogels to human skin, we gently and evenly pressed the plastic sheet onto a piece of porcine skin (about 10 cm by 3 cm) obtained from a local market and then peeled off the plastic sheet.

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REFERENCES

1. Drury, Jeanie L., and David J. Mooney. "Hydrogels for Tissue Engineering: Scaffold Design Variables and Applications." *Biomaterials*, vol. 24, no. 24, 2003, pp.

4337–4351., doi:10.1016/s0142-9612(03)00340-5.
2. Pille, Jan, *et al*. "General Strategy for Ordered Noncovalent Protein Assembly on Well-Defined Nanoscaffolds." *Biomacromolecules*, vol. 14, no. 12, 2013, pp. 4351–4359., doi:10.1021/bm401291u.
3. Arora, Bharti, *et al*. "Chemical Crosslinking: Role in Protein and Peptide Science." *Current Protein & Peptide Science*, vol. 18, no. 9, 2017, doi:10.2174/1389203717666160724202806.
4. Elvin, Christopher M., *et al*. "Synthesis and Properties of Crosslinked Recombinant pro-Resilin." *Nature*, vol. 437, no. 7061, 2005, pp. 999–1002., doi:10.1038/nature04085.
5. Schulz, André, *et al*. "Tyramine-Conjugated Alginate Hydrogels as a Platform for Bioactive Scaffolds." *Journal of Biomedical Materials Research Part A*, vol. 107, no. 1, 2018, pp. 114–121., doi:10.1002/jbm.a.36538.
6. Min, Kyoung-Ik, *et al*. "Covalent Self-Assembly and One-Step Photocrosslinking of Tyrosine-Rich Oligopeptides to Form Diverse Nanostructures." *Angewandte Chemie International Edition*, vol. 55, no. 24, 2016, pp. 6925–6928., doi:10.1002/anie.201601675.
7. Ma, Shen-Feng, *et al*. "Role of Tyrosine and Tryptophan in Chemically Modified Serum Albumin on Its Tissue Distribution." *Biological & Pharmaceutical Bulletin*, vol. 29, no. 9, 2006, pp. 1926–1930., doi:10.1248/bpb.29.1926.
8. Liu, Zhi-Ren, *et al*. "[2] Methylene Blue-Mediated Cross-Linking of Proteins to Double-Stranded RNA." *Methods in Enzymology RNA-Ligand Interactions Part B*, 2000, pp. 22–33., doi:10.1016/s0076-6879(00)18041-3.
9. Redmond, Robert W., and Irene E. Kochevar. "Medical Applications of Rose Bengal- and Riboflavin-Photosensitized Protein Crosslinking." *Photochemistry and Photobiology*, vol. 95, no. 5, 2019, pp. 1097–1115., doi:10.1111/php.13126.
10. Selvam, Susithra, and Ivy Sarkar. "Bile Salt Induced Solubilization of Methylene Blue: Study on Methylene Blue Fluorescence Properties and Molecular Mechanics Calculation." Journal of Pharmaceutical Analysis, vol. 7, no. 1, 2017, pp. 71–75., doi:10.1016/j.jpha.2016.07.006.
11. Sjöback, Robert, *et al*. "Absorption and Fluorescence Properties of Fluorescein." Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, vol. 51, no. 6, 1995, doi:10.1016/0584-8539(95)01421-p.