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Three-Dimensional Direct Laser Writing of PEGda Hydrogel Microstructures with Low Threshold Power using a Green Laser Beam

Haoyi Yu¹, Haibo Ding², Qiming Zhang³, Zhongze Gu² and Min Gu^{1,3}

Abstract

Three-dimensional (3D) direct laser writing (DLW) based on two-photon polymerisation (TPP) is an advanced technology for fabricating precise 3D hydrogel micro- and nanostructures for applications in biomedical engineering. Particularly, the use of visible lasers for the 3D DLW of hydrogels is advantageous because it enables high fabrication resolution and promotes wound healing. Polyethylene glycol diacrylate (PEGda) has been widely used in TPP fabrication owing to its high biocompatibility. However, the high laser power required in the 3D DLW of PEGda microstructures using a visible laser in a high-water-content environment limits its applications to only those below the biological laser power safety level. In this study, a formula for a TPP hydrogel based on 2-hydroxy-2-methylpropiophenone (HMPP) and PEGda was developed for the fabrication of 3D DLW microstructures at a low threshold power (0.1 nJ per laser pulse at a writing speed of $10 \mu\text{m}\cdot\text{s}^{-1}$) in a high-water-content environment (up to 79%) using a green laser beam (535 nm). This formula enables the fabrication of microstructures with micrometre fabrication resolution and high mechanical strength (megapascal level) and is suitable for the fabrication of water-responsive, shape-changing microstructures. These results will promote the utilisation of low-power 3D DLW for fabricating hydrogel microstructures using visible lasers in high-water-content environments.

Introduction

Three-dimensional (3D) direct laser writing (DLW) based on two-photon polymerisation (TPP)^{1,2} is a promising technique for the fabrication of novel 3D biocompatible scaffolds at the micrometre scale for biomedical engineering. It enables the creation of adaptable structures for applications such as cell culture³, artificial tissues⁴, wearable biosensors⁵, micromachines⁶, and neural tissue engineering⁷⁻⁹. In addition, 3D DLW enables the TPP

fabrication of biocompatible hydrogel microstructures, which provide a microenvironment similar to the extracellular matrix¹⁰. Hydrogel materials are sensitive to external stimuli, such as the variation in the water content¹¹, pH¹², and ion concentration¹³, which allow the temporal and spatial changes in the hydrogel microstructures to be controlled. Recently, various TPP hydrogels and microstructure designs have been developed based on unique two-photon-absorbing chromophores such as photoinitiators^{14,15} and precursors¹⁶⁻¹⁹.

The future development of 3D DLW for hydrogel scaffolds requires not only high biocompatibility, micrometre fabrication resolution²⁰, and high mechanical strength²¹, but also the incorporation of microstructures with biological tissues during fabrication. In this context,

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the laser safety threshold to prevent the detrimental effects of lasers on biological tissue is 1.5 nJ when a $100 \times \text{NA } 1.4$ objective is used at a $5 \mu\text{m}\cdot\text{s}^{-1}$ scanning speed in a high-water-content environment²². To satisfy the aforementioned conditions and specific requirements in biomedical engineering, several TPP hydrogel formulations have been developed²³. Among these, polyethylene glycol diacrylate (PEGda) is one of the most widely used hydrogels and has been approved by the FDA for several biomedical applications owing to its low toxicity and high biocompatibility²⁴. Recently, the TPP of PEGda was achieved using a near-infrared (810 nm) laser with a laser pulse energy of 1.37 nJ in a high-water-content environment²⁵. However, the TPP of PEGda under visible light in a high-water-content environment with low power has not been reported yet because of the lack of suitable photoinitiators. For specific biomedical applications, visible-light sources are highly preferred because of their accelerating effect in wound healing²⁶, low absorption efficiency in water²⁷, and weak interaction with biological tissues¹⁹. Therefore, it is necessary to develop novel formulas for TPP PEGda suitable for 3D DLW using a visible-light source in high-water-content environments.

Here, we report the 3D DLW of biocompatible TPP PEGda microstructures requiring only a low laser threshold power through green light illumination in a high-water-content environment. This was achieved using a formulation of a TPP hydrogel comprising 2-hydroxy-2-methylpropiophenone (HMPP) as the photoinitiator and PEGda as the precursor. HMPP was originally developed as a photoinitiator for single-photon ultraviolet polymerisation; it features a high water solubility and biocompatibility, and low cytotoxicity²⁸. It has not yet been utilised for the TPP of PEGda. HMPP enables TPP under 535 nm-wavelength illumination, which has been proven to accelerate wound healing²⁹ and offer finer resolution³⁰ compared with infra-red lasers. This TPP hydrogel formulation also exhibits a high photopolymerisation efficiency even at high water contents (up to 79%) and enables the use of laser threshold powers as low as microwatts (laser pulse energy of 0.1 nJ). In addition, the HMPP+PEGda hydrogel allows micrometre fabrication resolution, which is demonstrated here by the fabrication of cell-size woodpile microstructures with varied periodicities (1 μm resolution) at a high water content using the single-line fabrication method. The mechanical strength of the microstructures fabricated by the layer-by-layer method is also investigated. The mechanical strength of the microstructures makes our formulation a perfect candidate for fabricating neuron-inspired fractal structures for neural tissue engineering. These advantages allow the fabrication

of a microstructure named ‘octagons to squares’ to demonstrate the water-response property. This work hence opens the possibility for the low-power fabrication of hydrogels in a high-water-content environment by 3D DLW at a visible wavelength for future biomedical applications.

Results

The hydrogel microstructures were fabricated using a 3D DLW system comprising a femtosecond (fs) laser beam operating at a wavelength of 535 nm (Fig. 1a). The absorption spectra of the HMPP, PEGda, and HMPP+PEGda solutions were measured using a UV-visible spectrometer and are shown in Fig. 1b. There is an absorption peak at the wavelength of 247.5 nm, which corresponds to the TPP of the hydrogel around a wavelength of 535 nm. The TPP of the HMPP+PEGda hydrogel for the fabrication of the hydrogel microstructures is shown in Fig. 1c. The HMPP photoinitiator absorbed two-photon energy from the laser beam and generated radicals in the focussing region, leading to cross-linked polymerisation between the PEGda molecules and the formation of larger molecules. By tracing the designed structures with the piezo stage, microstructures were fabricated in the hydrogel.

The relationship between the polymerisation laser threshold power P_{th} and writing speed s was studied to experimentally characterise the polymerisation properties of the designed HMPP + PEGda hydrogel (Fig. 1d). The P_{th} of the HMPP + PEGda hydrogel with different ratios of water content ranged from 2.8 mW to 9.0 mW for s ranging from $1 \mu\text{m}\cdot\text{s}^{-1}$ to $100 \mu\text{m}\cdot\text{s}^{-1}$. These values correspond to laser pulse energies ranging from 0.056 nJ to 0.18 nJ, which are lower than the maximum safety laser threshold for applications involving biological samples. In addition, the ratio of water content had a significant effect on the polymerisation efficiency of the hydrogel. Fig. 1d shows that for a fixed scanning speed, the hydrogel with 79% water content required much higher laser energy to polymerise compared with the hydrogels with lower water contents.

In addition, the laser threshold power of the HMPP+PEGda hydrogel showed a power law relationship with the writing speed for shorter exposures (laser speed higher than $50 \mu\text{m}\cdot\text{s}^{-1}$, corresponding to an exposure time of 270 ms)³¹,

$$P_{th} \propto C \times s^{1/N} \quad (1)$$

where N is the nonlinearity exponent of the absorption for a specific photoinitiator, C is a coefficient related to the properties of the photoinitiators and precursors, and the

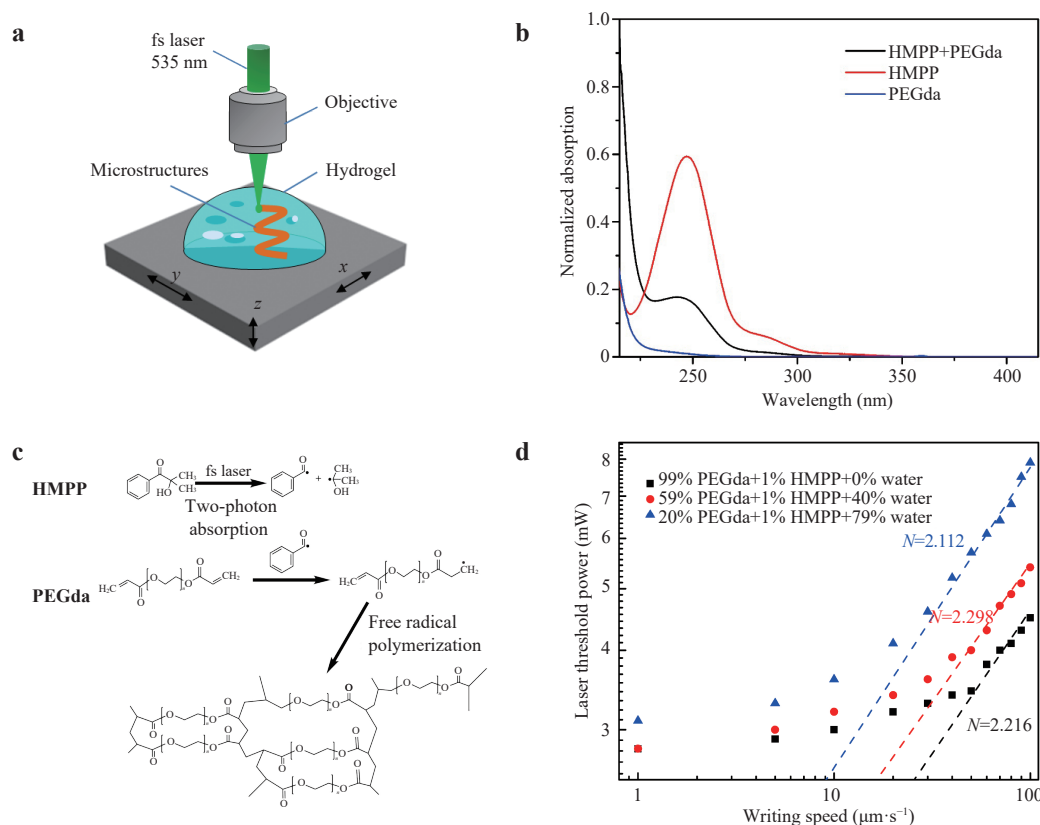
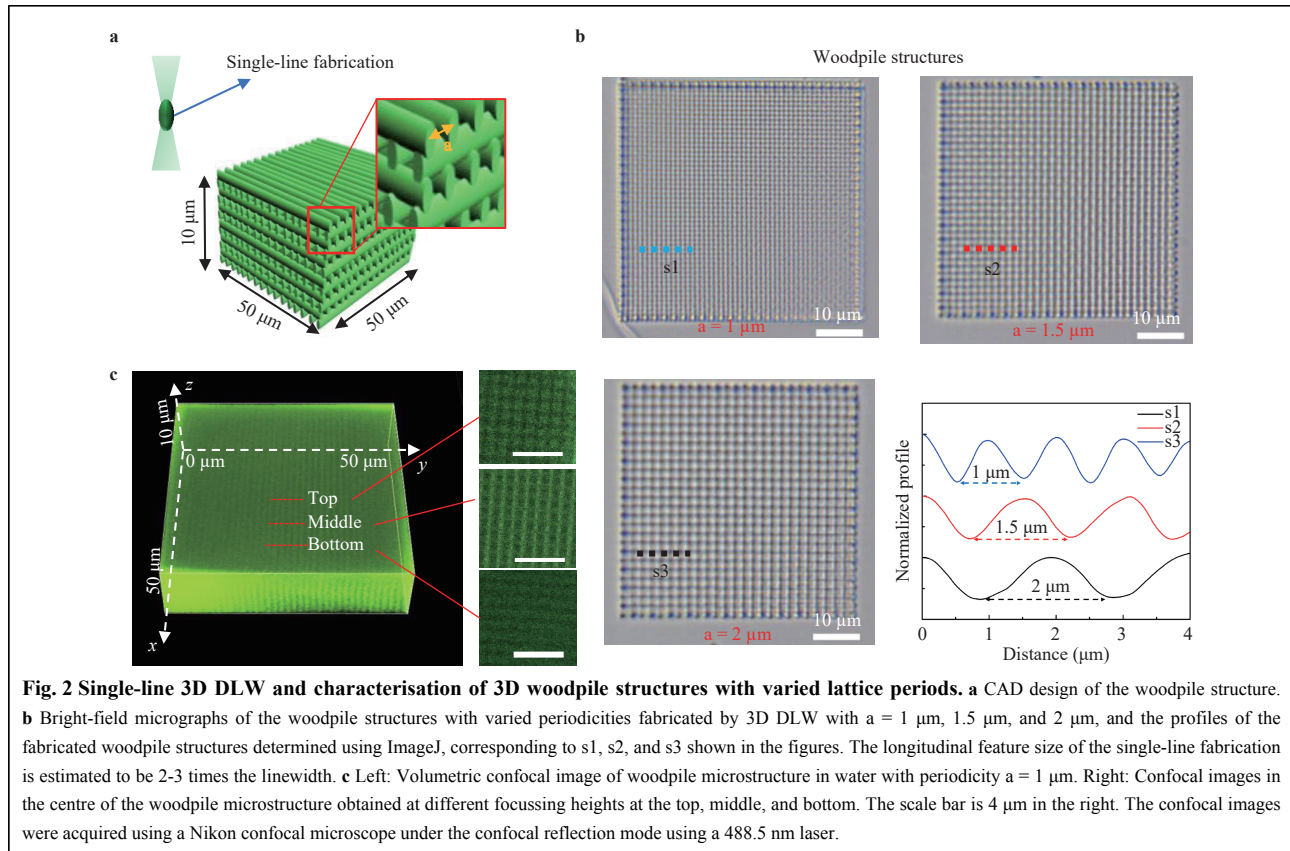


Fig. 1 HMPP+PEGda hydrogel for three-dimensional direct laser writing. **a** Schematic of the experimental setup for 3D DLW of the hydrogel microstructures. **b** Absorption spectra of HMPP, HMPP+PEGda, and PEGda solutions obtained using a UV-visible spectrometer. **c** Schematic of the TPP of the HMPP+PEGda hydrogel using 3D DLW. **d** TPP laser threshold power as a function of the writing speed for the HMPP+PEGda hydrogel for different ratios of water content.

laser threshold power P_{th} is defined as the minimum laser power needed to initiate polymerisation at a given writing speed, which can be obtained experimentally. By varying the water content in the hydrogel samples from 0% to 79%, N was determined to be 2 regardless of the water content ratio, while C decreased from 0.5786 to 0.8792. For longer exposures (laser writing speed lower than $50 \mu\text{m}\cdot\text{s}^{-1}$), P_{th} is significantly higher than the mathematical fit (straight dashed line) and becomes almost independent of the laser writing speed. This result can be attributed to a higher rate of radical generation in the TPP hydrogel compared to the rate of oxygen diffusion into the exposed volume, independent of the exposure time and laser writing speed. The influence of the water content on the polymerisation properties of the hydrogel can be understood through the polymerisation kinetics theory,³¹ according to which a higher water content ratio accelerates the movement of free radicals, necessitating a higher energy for polymerisation in the focal region. As a result, a high water content ratio requires a higher laser energy dose to form the polymerisation voxel in 3D DLW.

To demonstrate the micrometre fabrication capability, high mechanical strength, and water-response properties of the HMPP + PEGda hydrogel, various types of microstructures were designed and fabricated using the single-line fabrication and layer-by-layer fabrication methods. These experiments illustrate the versatility and potential of the hydrogel for fabricating 3D scaffolds for biomedical applications.

Using the single-line fabrication method, 3D woodpile microstructures were fabricated in an aqueous environment to illustrate the micrometre fabrication capability of the TPP hydrogel (Fig. 2a). As shown in Fig. 2b, the 3D woodpile structures fabricated with different periodicities a within a volume of $50 \times 50 \times 10 \mu\text{m}^3$ in the HMPP+PEGda hydrogel exhibited a highly uniform clearance similar to the size of the cell scaffold used in various works³². The normalised profiles of the woodpile structures are shown in Fig. 2b. The periods of the woodpile structures ranged from $1 \mu\text{m}$ to $2 \mu\text{m}$, corresponding to a two-line resolution of $1 \mu\text{m}$, and confirm the micrometre fabrication capability of the hydrogel. The 3D integrity and openness of the



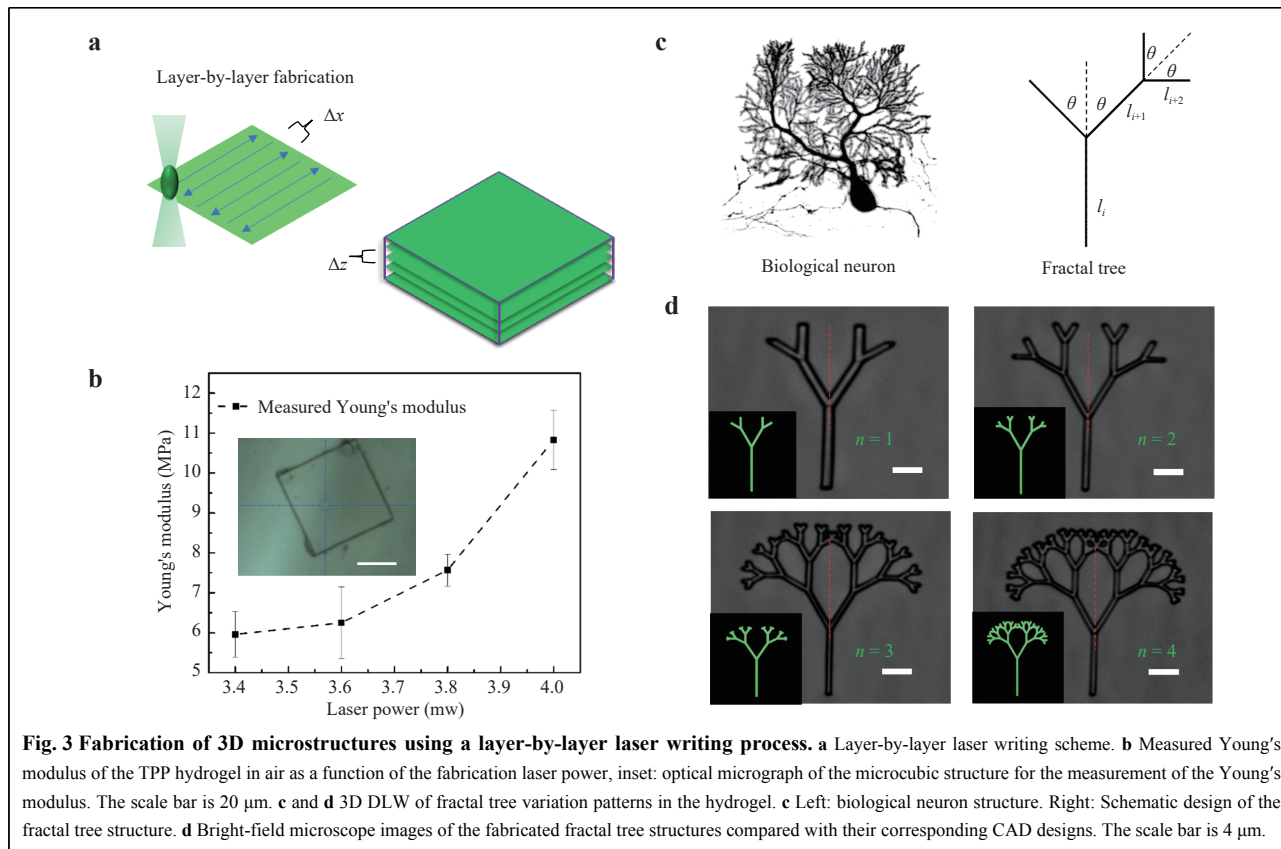
woodpile structures were verified by imaging the woodpile structure with a periodicity of $1 \mu\text{m}$ in water using confocal reflection mode microscopy. A green laser beam (488.5 nm) was used to scan and image the microstructure. The volumetric image of the woodpile microstructure is shown in Fig. 2c (left). Confocal images were also acquired at various focussing heights (top, middle, and bottom) in the centre of the microstructure. The images clearly show that the periodicity and the openness of the woodpile structure were maintained throughout the structure. Although the feature size from TPP fabrication using 535 nm light can be further reduced to approximately 190 nm in theory³⁰, it is very challenging in practice to reduce the microstructure feature size because of the instability of the microstructures and the optimised fabrication conditions required for specific fabrication tasks⁴.

Based on the polymerisation properties of the hydrogel, microcubic structures were fabricated by layer-by-layer fabrication (Fig. 3a, b). The microcubic structures were fabricated with a layer distance of 500 nm and size of $60 \times 60 \times 5 \mu\text{m}^3$ under various laser powers.

The hardness of the microcubic structures in air fabricated by different laser powers is shown in Fig. 3b, and a top-view transmissive optical image of a 3D cubic microstructure fabricated with a laser power of 3.6 mW, a

writing speed of $50 \mu\text{m} \cdot \text{s}^{-1}$, and a layer distance of 500 nm is shown in the inset. As shown in Fig. 3b, when the laser power was lower than 3.6 mW, 3D microcubic structures could not be fabricated because of the insufficient number of free radicals generated and subsequently, the inadequate polymerisation in the exposed area. When the laser power was higher than 4.0 mW, local explosions occurred because of the excessive heat within the exposed area of the resin. The explosions consequently caused bubbles and polymer decomposition. When the energy per unit area increased, more free radicals could be generated. Hence, the crosslink density of the polymer increased, and the obtained microstructure became harder to deform. As a result, the hardness of the hydrogel increased from approximately 6 MPa to 11 MPa.

In biomedical engineering, fractal structures are of great interest because of their unique geomaterial arrangement and mechanical properties³³. The fabrication of fractal structures for cell growth and cell proliferation has attracted much interest in biomedical engineering. Therefore, in this work, neuron-inspired fractal tree structures were designed and fabricated by layer-by-layer fabrication in the TPP hydrogel to further demonstrate its potential for future applications in neural tissue engineering. Fractal patterns have been found in various



natural systems, such as the bifurcation in lungs³⁴, vasculature networks³⁵, and biological neural networks³⁶ (as shown in Fig. 3c, left)³³.

The mathematical model of the fractal tree structure was based on Murray's laws³⁷, which describe a fractal branching network. The model includes the description of the geometrical parameters of a neural network (i.e., the length of each branch, its radius, and its branching angle). For a branch with a parent branch of length l_i and right-hand daughter branch of length l_{i+1} (at a branching angle of θ) that in turn has a daughter of length l_{i+2} branching off to the right at angle θ as shown in the right of Fig. 3c, Murray proposed the relation $l_{i+1} = 1/2 \times l_i$. By increasing the branch or fractal order n , different neuron-inspired fractal tree structures can be generated. Four neuron-inspired fractal tree structures were designed and fabricated in the experiment. As shown in Fig. 3d, we chose the branching angle to be 30° and the branching order n to range from 1 to 4. The optical images in Fig. 3d show that the fabricated microstructures maintained a very uniform geometry that is consistent with the computer-aided design (CAD) model.

Along with features such as a low laser threshold power, micrometre resolution, and relatively high mechanical strength, the TPP hydrogel also exhibits a water-responsive shape-memory effect due to its high water content ratio.

This feature was demonstrated by designing and fabricating a structure named 'octagons to squares' using 3D DLW and experimentally characterising its swelling and shrinking behaviours. An array of 4×4 identical octagons was arranged on a two-dimensional thin substrate (approximately 1 μm) and mutually connected with fixed joints (the red dots shown in Fig. 4a (right)). Each octagon has a size of $d = 40 \mu\text{m}$ defined as the distance between the two farthest free joints (blue dots shown in Fig. 4a), and a thickness of $z = 5.5 \mu\text{m}$. Owing to the unique dynamic change between the octagon and square shapes³⁶, the designed structures were able to shrink and swell, and thereby switch between the octagon and square shapes. The evaporation of water in the hydrogel microstructures caused the structures to shrink from octagons to squares. On the other hand, increasing the water content in the hydrogel microstructures caused the designed structures to swell from squares back to octagons.

The fabrication results and the water-responsive shape-memory effect are shown in Fig. 4b, c. The dynamic swelling and shrinking processes of the 3D hydrogel microstructure were quantitatively characterised by measuring the distance between the two farthest joints d (red arrow in Fig. 4b) as a function of time t . When water was added to the microstructure, the absorption of water in

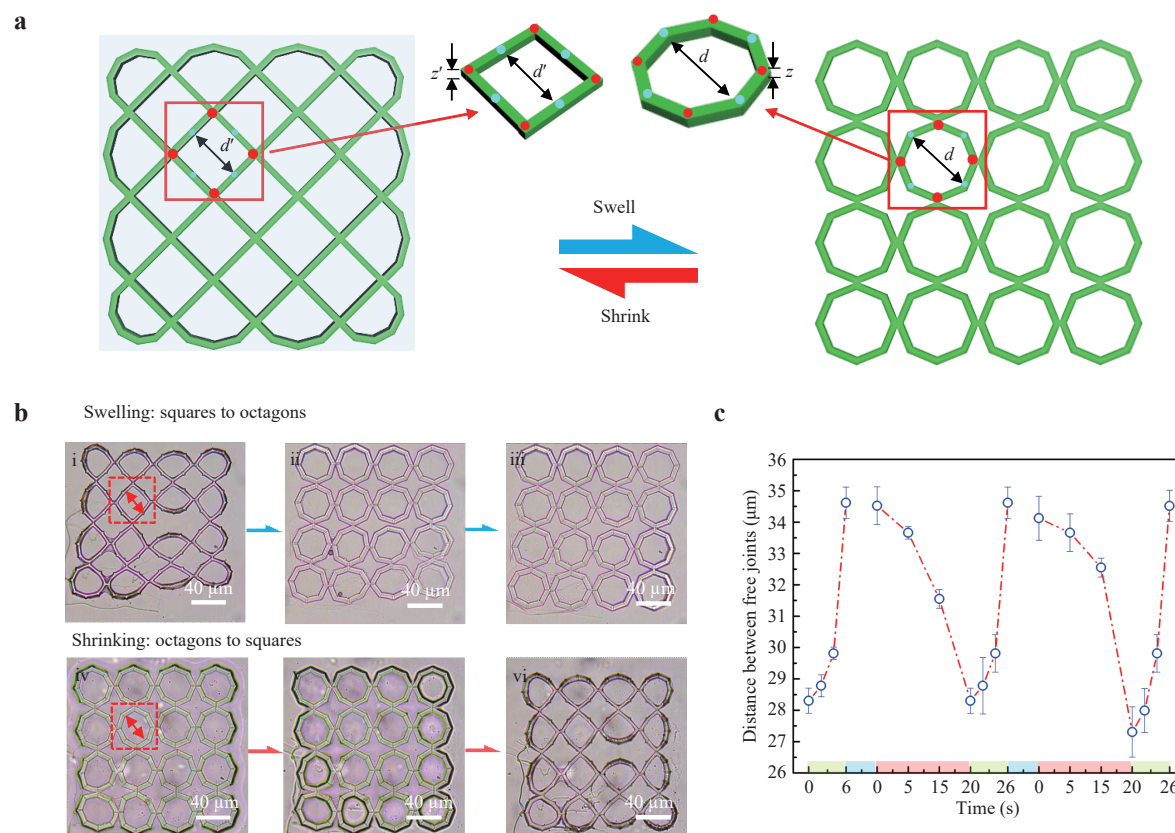


Fig. 4 3D DLW and characterisation of reversible shape-memory structure 'octagons to squares'. **a** An illustration of the shrinking and swelling processes of the shape-memory microstructure (red dots represent fixed joints; blue dots represent free joints) on a thin layer of substrate. **b** Fabrication results and experimental observations of the reversibility of 'octagons to squares'. **c** Quantitative study of the reversible behaviour of the 'octagons to squares' microstructure fabricated by 3D DLW. The distance between the free joints as a function of time was measured. The figure shows 2.5 cycles of the shape-change process, in which the green sections denote the swelling processes, red sections denote the shrinking processes, and the blue sections represent the saturated static state in which there is excessive water in the environment.

the hydrogel gave rise to an external force outward, relative to the fabricated structures that changed from squares to octagons (Fig. 4b (i-iii)). Quantitatively, it took the microstructure approximately 6 s to swell from a size of approximately 28.20 μm to 35.5 μm (shown in Fig. 5 with the green section, from $t = 0$ s to $t = 6$ s). When the microstructure was completely swollen, it remained in a saturated static state for a relatively long time (20–30 s) until the excess water in the microenvironment was reduced to a certain level (shown in Fig. 5 with the blue section). Compared with swelling, shrinking took a longer time (approximately 15 s). As shown in Fig. 4b (iv-vi), the evaporation of water in the microstructure resulted in an inward external force, and the octagons shrunk into squares. Therefore, the distance between the free joints in the octagons decreased from approximately 34.5 μm to 28.30 μm . The fabricated structure demonstrated a deformation ratio of 18% when swelling and shrinking. The deformation ratio is defined as the ratio of the largest

distance change to the original distance, that is, $\Delta d/d = 18\%$.

This water-responsive shape-memory effect was further studied quantitatively by measuring the shape change multiple times. Fig. 4c shows 2.5 cycles of swelling and shrinking. Our designed hydrogel microstructure maintained its integrity after the shape changes. This indicates a relatively high degree of reversibility in the microstructure (see Movie S1 in the supporting information for the complete shape-change process). Therefore, this 3D hydrogel microstructure exhibiting a water-responsive shape-memory effect is promising for various applications in micromachines and micromanipulation.

Conclusion

In conclusion, we have developed and characterised a TPP hydrogel based on HMPP and PEGda for 3D DLW. In contrast to traditional TPP hydrogels, our formulation is suitable for TPP under green laser illumination.

Furthermore, it not only enables micrometre fabrication resolution and high mechanical strength, but also exhibits high polymerisation efficiency and low threshold energy in a high-water-content environment. The influence of the water content ratio was also investigated theoretically and experimentally. The unique features revealed herein will allow the fabrication of neuron-inspired fractal microstructures that offer a potential platform for future applications in neuron tissue engineering. Moreover, micrometre-scale water-responsive microstructures induced by the dynamics of the water content were fabricated and characterised. With the development of various photoinitiators and hydrogel materials for biomedical engineering, expansion of the library of available materials is extremely vital to satisfy specific biomedical application requirements under varied fabrication conditions, such as the wavelength, fabrication speed, and laser power in a high-water-content environment. Therefore, the hydrogel material demonstrated in this work is a promising candidate for future 3D DLW applications in biomedical engineering, such as reversible microstructure platforms.

Materials and methods

Hydrogel synthesis. HMPP and PEGda (700 DA) were purchased from Sigma and used without any further processing. To synthesise the hydrogel, 1% volume ratio of the photoinitiator HMPP (Sigma) was added to 59% of the monomer PEGda (Sigma). Subsequently, 40% deionised water was added to the mixture. The entire mixture was then sonicated for approximately 60 s to obtain an evenly mixed solution. The ratio of the water content was varied from 0% to 79%. A further increase in the water content resulted in insufficient polymerisation and failure of the microstructures.

Fabrication and characterisation of the hydrogel microstructures. The experimental setup of the 3D DLW system is illustrated in Fig. 1a. A femtosecond laser beam operating at a wavelength of 535 nm (Fidelity), a pulse width of 270 fs, and a repetition rate of 50 MHz was steered by a combination of a 4f imaging system and 2D galvo mirrors (Thorlabs) into a 1.4 NA 100× oil immersion objective (Olympus). The beam delivery and power were controlled by an acoustic optical modulator (AOM) (Thorlabs). Within the focus of the laser beam, polymerisation occurred when the effective laser power exceeded the threshold of the TPP hydrogel. 3D microstructures were written by the translation of the sample on the piezoelectric nanotranslation stage (Physik Instrumente). After laser fabrication, the sample was developed with deionised water to wash out the unpolymerised parts at room temperature (20–22 °C).

Bright-field imaging of the microstructures was performed using an optical microscope (Olympus). The Young's modulus of the hydrogel microcubic structures was determined using the contact mode of a nanoindentation instrument (Bruker Multimode 8) with the relative method as the correction method. A 5 µm flat-end probe from Hyston was used. The loading force was a function of the applied time with a maximum force of 30 µN. The standard sample used in our experiment was polydimethylsiloxane (PDMS)-SOFT-1 (Tack-0 PDMS) 2.5 MPa supplied by Hyston, and the loading rate was 10⁻³ Hz. The confocal images were acquired using a confocal microscope (Nikon A1 HD25) under the confocal reflection mode using a 488.6 nm-wavelength laser.

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Author contributions

Haoyi Yu and Haibo Ding proposed the concept of using 3D direct laser writing to fabricate HMPP+PEGda hydrogel microstructures and its application in biomedical engineering. Haibo Ding proposed the formula of the photosensitive hydrogel and synthesised the material. Haibo Ding and Haoyi Yu designed and fabricated the hydrogel structures. Haoyi Yu performed the characterization of the material and interpreted the data. All authors participated in the discussions and contributed to the writing of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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