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TECHNICAL NOTE

Projection-based 3D bioprinting for hydrogel scaffold manufacturing

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Projection-based 3D bioprinting (PBP): a powerful method to fabricate 3D cellular structures

Three-dimensional (3D) bioprinting has played an important role in tissue engineering and regenerative medicine areas over the past decade [1]. Different from traditional cell cultures in Petri dishes, 3D bioprinting can build bionic structures with a better potential to become artificial organ substitutes [2–4]. With the development of photocurable biomaterials, the projection-based 3D printing method has been successfully applied in biological research [5, 6]. It possesses significant advantages in precision and speed due to its unique manufacturing principle. On the one hand, light-dependent solidification allows it to build models with resolutions around 50 μm (theoretically a minimum of 1 μm) [7]. On the other hand, its surface-to-entity printing process provides duplicates in batches without extra time requirement [8]. Therefore, it has attracted the attention of many researches devoted to the field of 3D cell culture.

Excellent printing precision

The printing precision of PBP is determined by the optical parameters of the projector. The digital micromirror device (DMD) is a key component of PBP, which consists of an array of micromirrors that can rotate independently. Each micromirror corresponds to a single pixel of PBP. Theoretically, the minimum pixel size can reach 1 μm. Considering the printable area and the financial cost, the pixel size in common PBP devices is around 50 μm. Compared with traditional extrusion-based bioprinting in which the diameter of fiber is over 200 μm, PBP has significant advantages in building microstructures.

High printing speed

The duration of projection-based printing depends on only two factors: the crosslinking time for each layer, and the number of layers. The photo-crosslinking reaction is rapid: photocurable biomaterials need about 10–60 s to finish crosslinking. A centimeter-scale model usually needs only less than an hour to be constructed. Importantly, benefiting from the unique surface-to-entity printing principle, the duration of PBP is independent of volume. When different models are printed in parallel, the duration only depends on the tallest model. This allows PBP to print models with duplicates but without extra time consumption, which greatly improves its printing efficiency.

Ideal for medical applications

The high printing resolution of PBP ensures the precision of the fabricated microstructures. Not only it allows researchers to build smaller models for medical applications, but it is also a powerful method to study the mechanism of cell behavior guided by microstructure. Furthermore, most of the achievements in biology and medicine are based on statistics, which requires a large number of repeated experiments; PBP is good at providing consistent duplicates in large quantities for such experiments. Above all, PBP has great potential to become the mainstream method of bioprinting.

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Why has projection-based bioprinting not been widely applied?

Limited supply of professional devices and stable materials

Building a projection-based bioprinter is not an easy task. It requires a team of interdisciplinary experts, especially those in optics, biology and engineering. Meanwhile, most of the commercialized printing devices are too expensive to become as common as microscopes in laboratories. Besides, synthesizing photocurable biomaterials requires skilled operators and professional equipment. Only experienced operators or automated production lines can guarantee consistent material properties between different batches. Also, unstable material properties lead to unreliable follow-up experimental results.

No standard operating procedures

Projection-based bioprinting includes many process parameters that can greatly influence the results. The study of printability is a relevant professional topic in engineering, and researchers with biological or medical background will have hardly enough professional knowledge or aspiration to deal with this field. In fact, all that potential users need is a not-too-optimal but simple and clear operating procedure. However, reasonable operating procedures are associated not only with device performance, but also with material properties, which two factors are set by the manufacturing company; costumers will have to test the compatibility of materials and devices on their own.

Multiple problem-prone details during printing

The printing process consists of several steps, and any unreasonable aspect of operation can cause failure. There are many specific challenges, such as the preparation of aseptic bioink with both good printability and biocompatibility, and ensuring that the prints stick to the platform during printing instead of falling off or sticking to the tank bottom, preventing the overexposure of deep-hole structures, or seeding cells adequately and even onto a 3D scaffold. To deal with these problems proficiently, researchers need months of repeated tests to gain the necessary skills and experience [9]. If any of these problems is not solved during printing, no satisfactory result will be achieved and all previous attempts will seem meaningless.

Lack of systematic training of operators

Published papers are the most important source to acquire relevant knowledge and information about existing technologies. However, most of the published papers on projection-based bioprinting only focus on the application significance or esoteric manufacturing theories, which offer less help to a novice to start a practical operation. Due to the differences between devices and biomaterials, the information provided on the materials or experimental procedure is also insufficient to obtain a reproducible result. As an emerging application, projection-based bioprinting still has a long way to become friendly toward researchers with non-engineering backgrounds.

What can be learned from this technical note about projection-based bioprinting?

Briefly, by following this note, beginners will have a systematic understanding of the projection-based printing process and can quickly achieve satisfactory prints that are useful for simple applications. In response to the questions raised above, we first introduce the brief basics of a projection-based bioprinter and photocurable biomaterials. Then, we offer a standard protocol from biomaterial preparation, printing operation, and post-processing to cell seeding. We attempt to take a beginner's view when describing the issues in detail. Finally, we demonstrate a vessel model to introduce the reader into the overview a whole application of projection-based bioprinting. We hope to lower the difficulty for researchers with non-engineering backgrounds of using this powerful method in their professional studies.

Introduction of components for PBP: materials, devices and printing principle

Photocurable biomaterials

Photo-crosslinking is the fundamental requirement for materials used in PBP. Besides, biocompatibility is necessary when applied for medical purposes. With the development of biomaterials, many choices of photocurable biomaterials have become available, such as polyethylene glycol diacrylate (PEGDA), hyaluronic acid methacryloyl (HAMA), and silk methacryloyl (SILKMA). The idea of inventing such materials is simple: gifting a natural biomaterial with the ability of crosslinking. In general, gelatin methacryloyl (GelMA) is the most frequent choice due to its excellent formability and biocompatibility.

GelMA, which is a modified form of gelatin, is a typical kind of photocurable biomaterial. It is synthesized from gelatin and methacrylate under controlled temperature and pH conditions. With the help of photoinitiator, it can turn from liquid into gel state when exposed to light with certain wavelength. The substitution degree is the most important parameter that indicates how many amino groups on gelatin

are substituted by double bond groups. The more the substituted amino groups, the better formability will be achieved. However, biocompatibility and formability are contradictory; thus, we usually need to achieve balance between them according to the experimental purpose. On this technical note, GelMA (EFL, EFL-GM-90, Suzhou, China) with a substitution degree of 90% is a commonly used material for scaffold printing.

Projection-based 3D bioprinter

A projection-based 3D bioprinter essentially consists of a projection unit, a motion unit and a material storage unit. The projection unit includes a 405-nm wavelength projector and related optical components, and it is wirelessly connected to a computer and will project the image of each model slice onto the forming area. The motion unit is composed of a printing platform with only one degree of freedom. It will rise to the next position after the printing of the current layer is over. The material storage unit consists of a bioink tank and a temperature controlling system. The bioink tank has a transparent bottom to let the image project through. The temperature controlling system warms the material to 37 °C to prevent it from condensation. On this technical note, a desktop projection-based bioprinter (EFL-BP-8601P, Yongqinquan Intelligent Equipment Co., Ltd., Suzhou, China) is an example that was employed to build GelMA hydrogel scaffolds.

Printing principle

Projection-based 3D bioprinting can be summarized as a surface-to-entity forming method. As the printing process begins, the platform firstly goes down and submerges under the liquid material surface, leaving a thin gap between the tank bottoms. This gap, when filled with materials, has the same thickness with each digital slice. Then, the projector sends the image of the first slice through the transparent bottom, illuminating the materials within the image boundary. In a short period, the photocurable liquid material crosslinks and turns into gel state. Subsequently, the platform rises to leave another gap between the first print and the tank bottom. Then, the image of the next slice is projected. The special design of the hydrophobic bottom guarantees that the new print will stick to the platform or the former slice. Finally, as the cycle continues, the whole model will be printed layer by layer. Obviously, with the same process parameters, the printing duration is only determined by the height of the models, and it has nothing to do with the area of each image. Therefore, the PBP method is particularly good at manufacturing large quantities of consistent sample copies.

Workflow

Step I Hydrogel preparation

The GelMA ink for PBP scaffolds consists of three ingredients: GelMA powder, photoinitiator and photoabsorber. As mentioned above, we recommend GelMA with 90% degrees of substitution and 15% (w/v) concentration to beginners. Higher degrees of substitution and concentration will significantly increase the modulus of crosslinked hydrogel and improve printability; however, this will correspondingly decrease the biocompatibility. Lithium acylphosphinate (LAP), a safe and efficient photoinitiator with an absorption spectrum from 365 to 405 nm, will convert light energy into chemical energy and stimulate the crosslinking reaction during printing. Levels of 0.1–0.5% (w/v) LAP are sufficient for PBP, and 0.5% is recommended for scaffold printing. Citrine food coloring is used as a non-toxic photoabsorber with a concentration of 0.5% (w/v) to improve printing quality.

Phosphate buffer solution (PBS) is employed to dissolve these ingredients. There is no requirement for the order in which they should be added, but every ingredient must be wetted by cold PBS. Then, the mixture is heated to 60 °C and stirred for about 30 min until fully dissolved. Bubbles and other impurities are removed by centrifugation (2 min, 1000 rpm). Otherwise, these impurities can form countless reflective interfaces and greatly decrease the printing quality. A well-prepared GelMA ink is an orange clear liquid. The prepared GelMA ink can be stored at 4 °C for up to a week or −20 °C for no more than a month. It is worth noticing that, when using ink stored at −20 °C, it should be firstly returned to normal temperature in cold water until no ice can be observed but keeping it in gel state, then heated to 37 °C to a liquid state.

Step II Scaffold printing

The printing process is controlled by a computer while the operator only needs to fill the materials, set the parameters, and collect the prints. However, setting the parameters reasonably is not an easy task. Here, we provide some practical directions. First of all, models can be constructed by many types of engineering software, like Solidworks, or obtained from a model library. The user needs to make sure that the files are in STL format. Then, the orientation of models should be edited technically in the printing software, for it can greatly influence the printing duration and shape accuracy. As mentioned in the printing principle section, the duration of PBP is only related to the number of layers when the exposure time of each layer is fixed by the material features. For example, printing of a tubular model placed vertically (diameter: 2 mm, length: 20 mm, Fig. 1 STEP II) will consume 10 times of that placed horizontally. However, the accuracy of projection-based printing is quite different when it is in

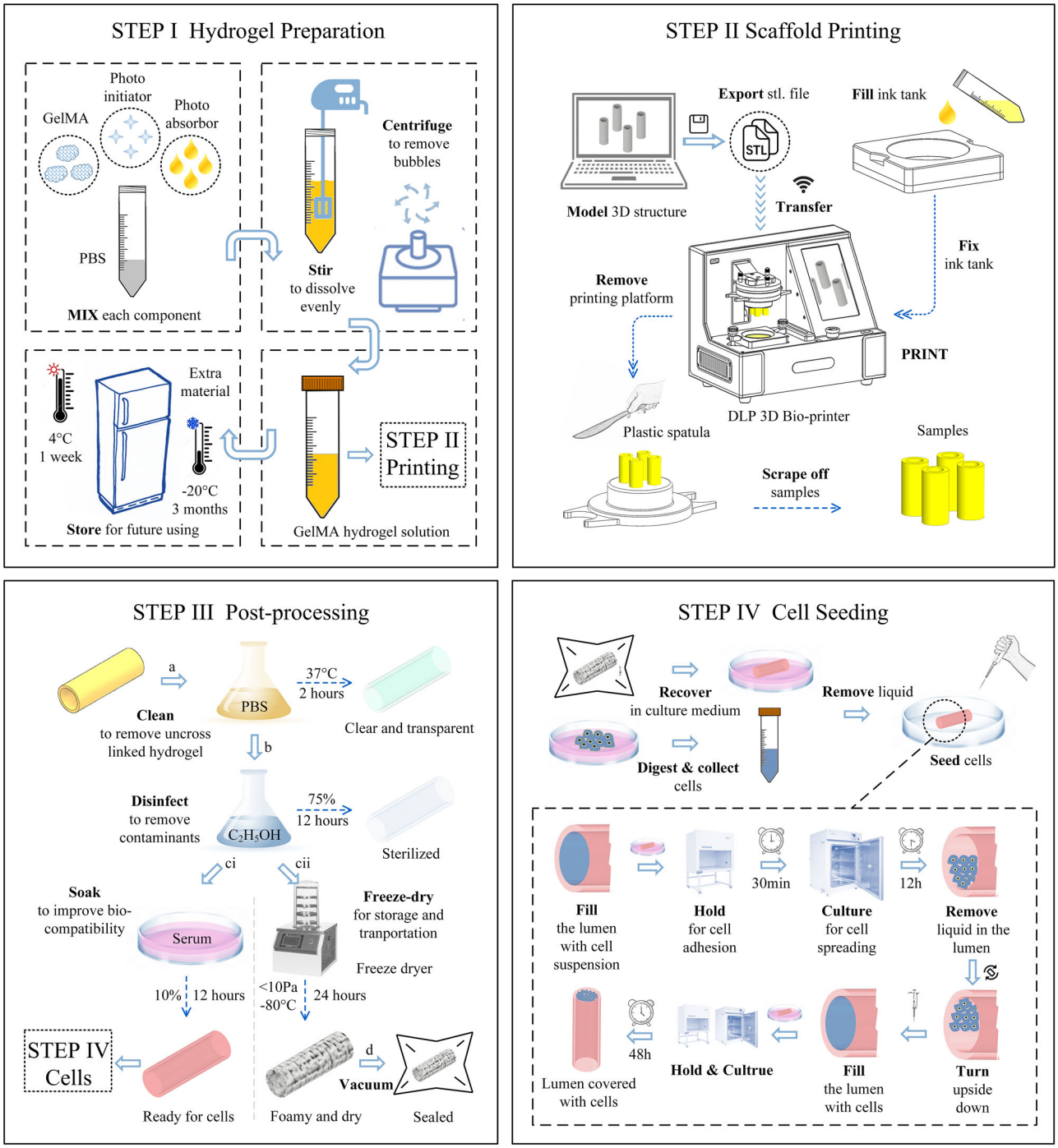

Fig. 1 Workflow of projection-based bioprinting

the *x*–*y* plane or along the *z* direction. Influenced by light transmission and the requirement of stable bonding between layers, printing accuracy along the *z* direction can be 2–3 times worse than that in the *x*–*y* plane. Therefore, if accuracy is taken into consideration, the tubular model must be placed vertically to achieve the best roundness of both the inner and outer diameter. Meanwhile, vertically placed tubes occupy a smaller area on the platform, so that we can set more duplicates in one printing. This arrangement will provide the best accuracy and highly consistent tubular structures in mass.

Although the printing platform and the ink bottom are specially treated, slender structures with a small bottom may still fall from the platform during printing. To prevent this phenomenon, we recommend a technique called "self-coating."

Specifically, before fixing the platform to the printer, a thin layer of GelMA ink may be applied to the platform and crosslinked. This layer will significantly strengthen the bond between the prints and the platform, so that models with large aspect ratios can be printed more stably.

Step III Post-processing
Post-processing is a very important but often ignored step in projection-based bioprinting. Cleaning is the first and most important operation to preserve the printing quality. It is easy to imagine that the newly printed tubular models will be filled with uncrosslinked GelMA ink in its cavity. If this uncrosslinked material is not removed in time, sunlight or indoor lighting will slowly make it to crosslink, which will in turn block the cavity. This could happen on other prints and lead to any detailed features on them covered and lost. Heated PBS is the best cleaning fluid and is a good solvent for GelMA, as its similar osmotic pressure will not cause model deformation. Disinfection is another indispensable step. Prints should be immersed in 75% medical-grade alcohol overnight, which operation will cause the prints to shrink, but this phenomenon is reversible. Sterilized prints can be freeze-dried for long-term storage and transportation, or they can be immediately immersed in serum for cell seeding.

Step IV Cell seeding
In short, cell seeding is to evenly cover the expected surface of scaffolds with cells. However, cell adhesion is affected by gravity. For many scaffolds with complex shapes, it is not easy to achieve uniformly distributed cells through a single seeding step. For example, a tubular model needs at least two seeding cycles to fully cover its lumen. The higher the number of seeding times, the more uniform the cell adhesion will be. Notably though, after the cell suspension is added onto the target scaffold surface, it takes at least half an hour for cells to establish bonding with the scaffold. Thus, any movement or shaking should be avoided within half an hour after seeding. Once the cells have been successfully seeded onto the scaffolds, they can be further cultured with traditional methods.

Examples

Experimental methods

As a demonstration, a projection-based bioprinted vessel scaffold was cultured with human umbilical vein endothelial cells (HUVECs). The vessel model was designed as a hollow cylinder (length: 10 mm, inner diameter: 1.2 mm, outer diameter: 2 mm) using Inventor (Autodesk). GelMA bio-ink was prepared with 15% (w/v) EFL-GM-90, 0.5% citrine and 0.5% LAP. The exposure time for each layer was set as 25 s. 1×10^6 HUVECs/mL were prepared in common culture medium for cell seeding. Other operations such as platform coating, post-processing and cell seeding were the same as introduced in the WORKFLOW section. The cell-seeded scaffolds were then cultured for 4 days and observed with confocal fluorescence microscope after immunofluorescence staining.

Experimental results

As shown in Fig. 2a, the designed model can be successfully printed in 13 duplicates within a single printing process. The yellow bottom is the anti-dropping GelMA coated on the printing platform. It is easy to find that the hollow vessels are still filled with uncrosslinked bioink in the lumen. After post-processing, the yellow color in the models becomes much lighter, and the lumen also becomes unobstructed. These results indicate that the printing was successful. Here, when a different type of hydrogel material is used, we may get two different undesirable results. One is that no prints are left after printing, which is caused by underexposure, so an extra period of exposure for each layer should be added. Another is

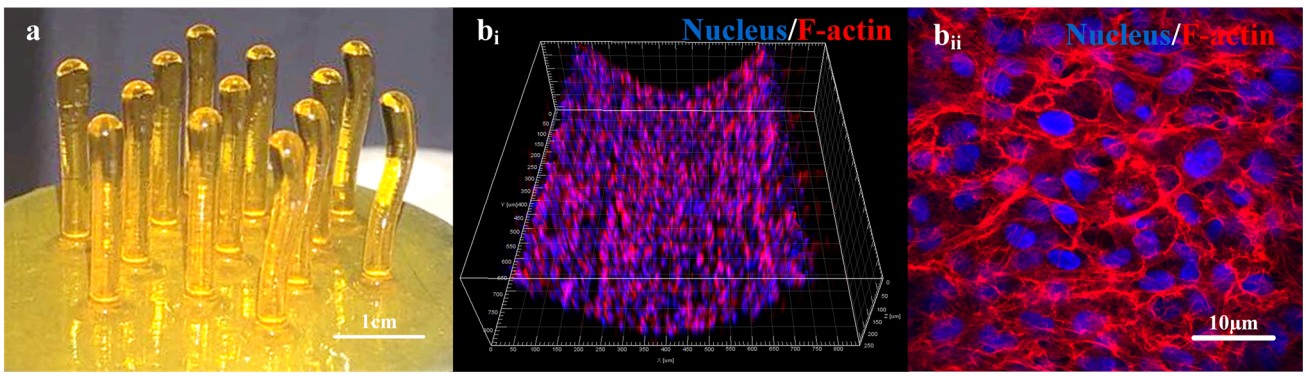

Fig. 2 Vessel scaffolds with HUVECs: **a** Projection-based bioprinted vessel models with consistent duplicates; **b** Three-dimensional confocal image of part of the vessel model with HUVECs

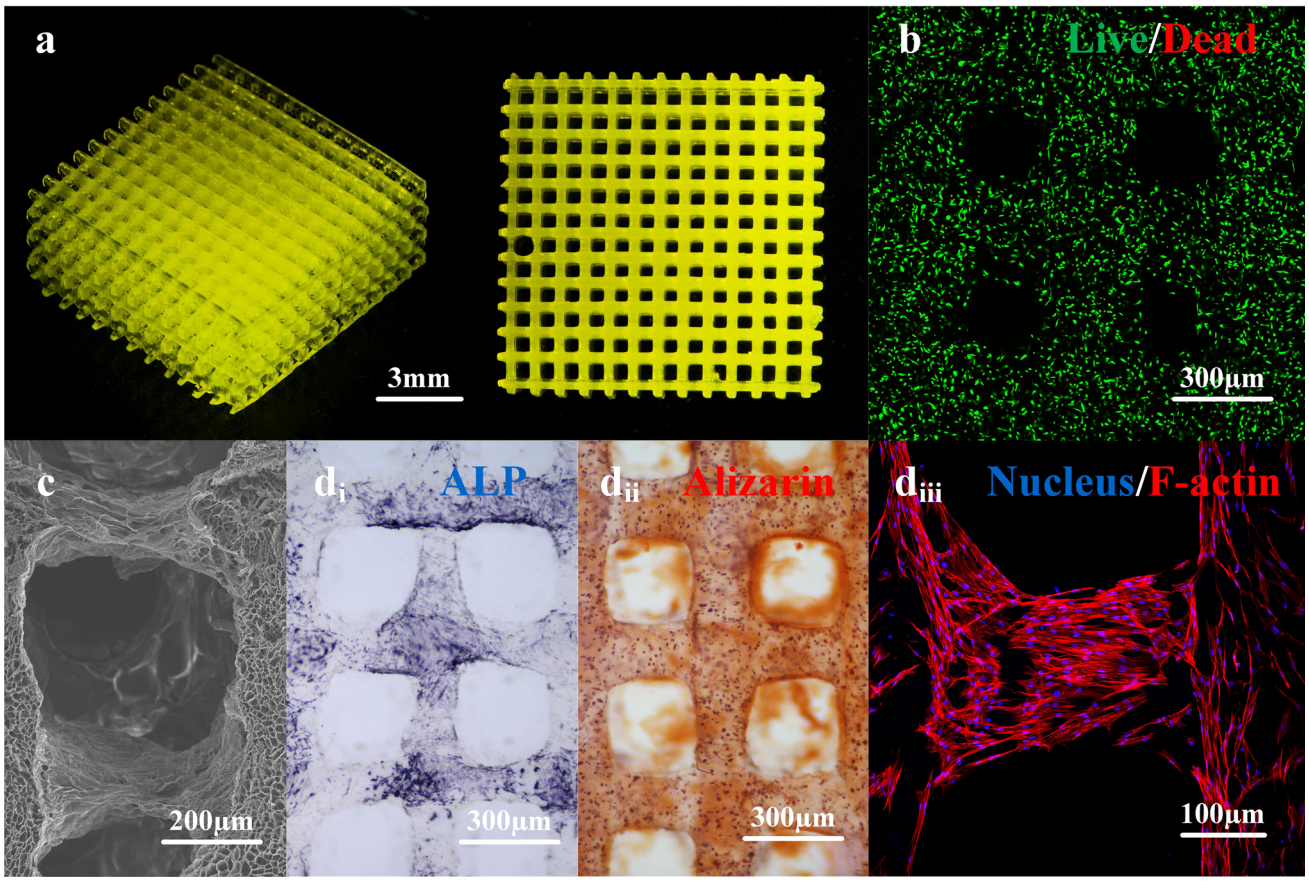

Fig. 3 Three-dimensional grid structure with functionalized BMSCs: **a** Image of the projection-based bioprinted grid scaffolds; **b** Live/dead staining of the seeded BMSCs on day 1; **c** Scanning electron microscopy of the scaffold; **d** Characterization of the functions of BMSCs cultured on the scaffolds

that even the post-processing has been conducted, the lumen is still blocked by crosslinked hydrogel. Consequently, the concentration of photoabsorber should be slightly increased.

Figure 2b shows that HUVECs can spread evenly and fully cover the inner surface of the lumen, outlining its cylindrical three-dimensional shape. Under high magnification, endothelial cells are tightly connected to each other, forming a single endothelial layer.

Additionally, a porous 3D grid structure with bone marrow mesenchymal stem cells (BMSCs) is shown in Fig. 3 to demonstrate the bio-functionalization potential of projection-based bioprinted scaffolds. Smooth holes with less than 400 μm in diameter run through the whole cubic scaffold to provide adequate material exchange (Fig. 3a). After one day of culture, almost no dead cells can be observed (Fig. 3b). The scanning electron microscopy results show the micro- to nano-scale holes distributed in the walls of the structure (Fig. 3c). The characterization of BMSCs' functionalization shows that cells spread well with desirable morphology, and ossification can be detected (Fig. 3d).

Discussion

Nowadays, 3D bioprinting technologies are developing at an immense speed, and many projection-based 3D bioprinting methods for cell printing have been reported to date. The question is why we still rely on this technique to print scaffolds.

The first answer is the contradiction between biocompatibility and formability. Photocurable biomaterials with good formability require more crosslinking points to form the entire molecular network faster. However, this inevitably increases the tightness of this network and reduces the rate of nutrient exchange; cells will be wrapped in hard nutrient-poor cages. On the other hand, reducing the concentration and substitution rate of biomaterial will improve its biocompatibility but sacrifices its formability, because it will take much longer to print or it will even fail to form a gel. Usually, there is only a very small intersection window between the ideal ranges of these parameters; thus, neither of them can be optimally adjusted. Printing scaffolds, however, can avoid this problem. Cells laden on a scaffold can actually grow first in a two-dimensional (2D) environment while being directly

exposed to fluid. The 3D distribution of cells is achieved by the design of the 3D scaffold surface. Therefore, the requirements for printing and cell viability can be separated, which allows researchers to build much more complicated tissue models.

The second answer is that projection-based bioprinting of scaffolds can greatly reduce the complexity and increase the printing success rate of the scaffold. Compared to cell printing, scaffold printing and cell seeding are separated both in the time and space dimensions. For cell printing, one must prepare well-cultured cells, bioink with the optimal properties, a working printer and other necessary elements at the same time and at same place. This will significantly increase the risk of errors during the process, which may cause delays and have a large impact on cell viability. Moreover, complicated processes during bioprinting increase the probability of pollution, which is not a scientific but rather a practical engineering problem. However, scaffold printing makes the task much simpler, as it enables the printing of scaffolds independently from cell culturing issues. At the same time, post-processing, which cannot be conducted with living cells present, effectively improves the quality and precision of models. It has obvious advantages in print stability and success rate. What is more, the feasibility of freeze-dry preservation ensures long-term storage and transportation of the print, which makes standardized clinical applications easier to implement.

Overall, cell printing and scaffold printing both have their distinct advantages. For researchers with no engineering background, we consider scaffold printing as a simpler method to apply in the fields of regenerative medicine and drug screening.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study does not contain any work related to human or animal subjects performed by any of the authors.

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