Bio-Design and Manufacturing (2021) 4:149–153
https://doi.org/10.1007/s42242-020-00096-2

TECHNICAL NOTE

Facile 3D cell culture protocol based on photocurable hydrogels

Mingjun Xie[1,2,3] · Yating Zheng[3] · Qing Gao[1,2,3] · Yong He[1,2,3]

Received: 8 August 2020 / Accepted: 17 August 2020 / Published online: 1 September 2020

Introduction

Why 3D cell culture urgently needed?

Tissue engineering has extremely influenced the development process of basic biological studies and biomedical technology. For quite a long time, the vast majority of these researches have been relying on the experimental results of conventional two-dimensional (2D) cell culture in flask, petri dish or well plate. However, these 2D culture results could be very different with or even totally opposite to the actual situations in vivo where cells grow inside a 3D extracellular matrix (ECM). Therefore, 3D cell culture has been playing a more and more significant role because of its higher accuracy and authenticity [1–4].

What methods of 3D cell culture have been used?

So far, there have been three main classes of 3D cell culture method, namely *low-adhesion reunion culture, suspension drop culture, and hydrogel encapsulating culture*. In low-adhesion reunion culture method, cells are usually seeded on the surface of petri dish with low adhesion, so that the cells tend to aggregate with each other to form 3D particles rather than attach or spread. In suspension drop culture method, cell suspension droplets are hanged upside down on the basement material. Cells would settle down with gravity effect and gather at the bottom of droplets to form 3D cell microspheres. In hydrogel encapsulating culture method, cells are mixed with hydrogel precursor. After that, with some formation approaches, precursor can be formed into specific 3D shapes with certain intensity and provide a 3D growing environment for the encapsulated cells. Compared with the two former methods, the hydrogel can be combined with many 3D bioprinting methods to form special 3D structures [5–10]. What's more, an ocean of micropores is produced during the cross-linking treatment. The encapsulated cells can acquire enough space for material exchanging; thanks to the hydrogel network, so that the 3D samples can be designed with much larger general sizes. Therefore, hydrogel encapsulating culture method is more closed to the actual conditions in vivo and owns more potential to be extended in a large scale.

Why 3D cell culture not popular so far?

In our opinion, if the operation approach of 3D culture is simple as 2D culture, it could be widely used by most of the researchers. As we all know, with a long-term development, 2D cell culture has formed a full set of nearly perfect strategy from culture to postprocess and characterization. *However, in 3D cell culture process, researchers are confronted with difficulties to build 3D environment in a simple way*. Hydrogels with higher biocompatibility usually have weaker strength, which makes them difficult to operate. Moreover, for subsequent characterization, it should be convenient to collect the cells inside hydrogels and carry out a series of biological examinations.

Start a simple and effective 3D culture journey

In this technical note, we would like to share a brand-new protocol of 3D cell culture based on photocurable hydrogels, which is as convenient and feasible as conventional 2D cell culture from culture system establishment to cell collection without injury and relevant characterizations (Fig. 1). This novel strategy consists of four main steps: bioink preparation, culturing in 3D environment, cells collection, and biological testing.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s42242-020-00096-2) contains supplementary material, which is available to authorized users.

✉ Yong He
yongqin@zju.edu.cn

1 State Key Laboratory of Fluid Power and Mechatronic Systems, School of Mechanical Engineering, Zhejiang University, Hangzhou 310027, China

2 Key Laboratory of 3D Printing Process and Equipment of Zhejiang Province, School of Mechanical Engineering, Zhejiang University, Hangzhou 310027, China

3 Engineering for Life Group (EFL), Suzhou 215101, China

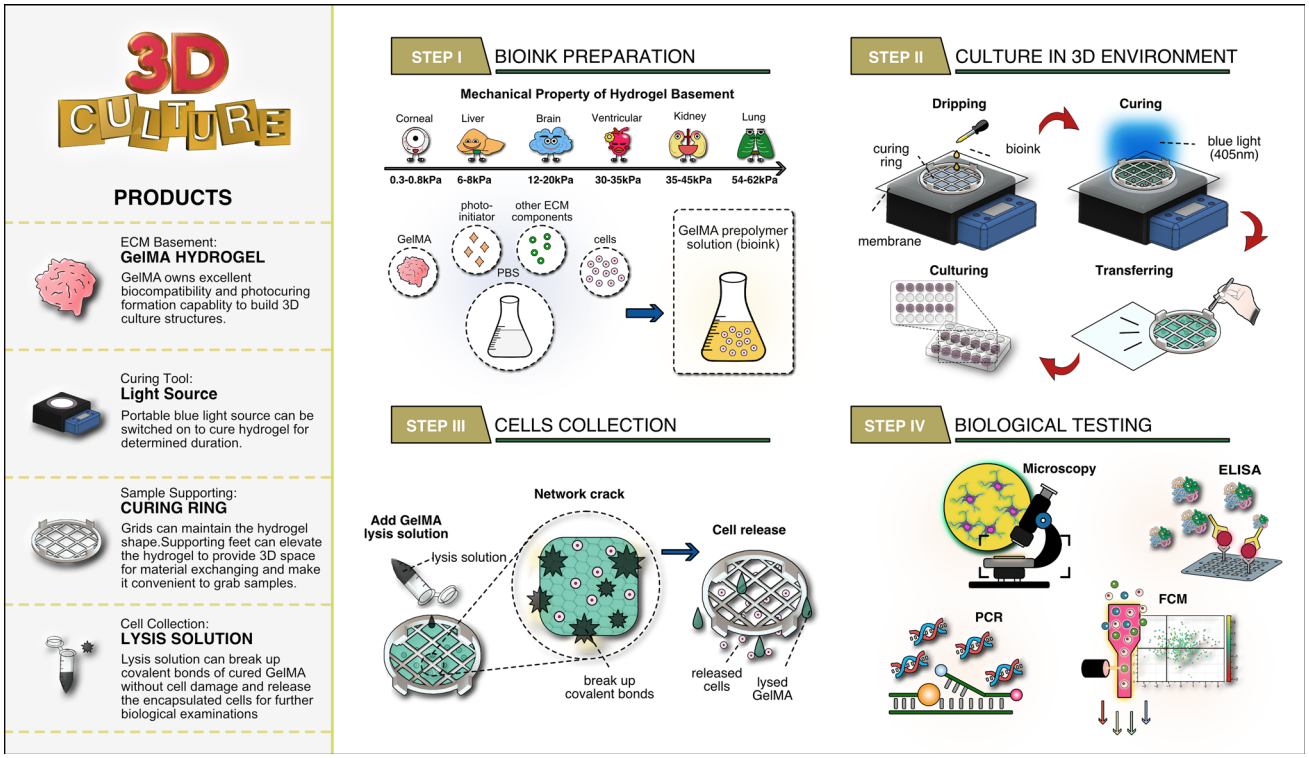

Fig. 1 Workflow sketch of the facile 3D cell culture solution with GelMA

Here, we designed a series of devices to accelerate this 3D culture process. A series of gelatin methacryloyl (GelMA) hydrogels which can match the environment of different organs from liver to cartilage, curing rings, timing blue light source, and GelMA lysis solution can be chosen for carrying out the proposed strategy to meet different 3D cell culture requirements. We examined and confirmed the feasibility of this protocol by culturing and characterizing several kinds of cells in this way. In summary, we believe this facile 3D cell culture workflow based on photocurable hydrogels, especially GelMA, will potentially become the mainstream in biomedical research.

Workflow

Step I: Bioink preparation

In actual organisms, different tissues own matrix with specific components and strength to achieve a series of biological functions. Therefore, in terms of 3D cell culturing, the first factor needs to be considered is the selection of ECM. An appropriate ECM for 3D culture in vitro owns three main requirements: biocompatible hydrogel basement, suitable strength, and other necessary ECM components. Here, GelMA hydrogel is chosen as the ECM basement for 3D culture in vitro because of its excellent biocompatibility and formation capability [11, 12]. Select GelMA hydrogel with suitable degrees of substitution and other necessary components according to the actual tissue in vivo. For example, if researchers want to culture liver cells in 3D ECM, they need to select appropriate GelMA type which can achieve 6–8 kPa tensile modulus after being cured [13]. Importantly, the acellular matrix of liver tissue is needed to be added into GelMA prepolymer solution. In terms of cartilage cells, researchers need to add enough hyaluronic acid to realize relevant biological functions. After the selection of GelMA and other components, GelMA prepolymer solution is prepared by dissolving the freeze-dried GelMA in phosphate buffer saline (PBS) at suitable concentration containing photo-initiator lithium phenyl-2, 4, 6-trimethylbenzoylphosphinate (LAP) and the chosen additions. Cells are detached from flasks and resuspended with the prepared solution to form the bioink.

Step II: Culturing in 3D environment

In culture process, for one thing, 3D cell culture needs all-round material exchanging space, so samples have to float inside the culturing medium rather than simply sink to the bottom of petri dish. For another, cross-linked GelMA hydrogel, especially with lower degree of substitution, has lower mechanical strength though it has higher biocompatibility. These 3D samples are always too soft to maintain the initial shapes during culturing and to manipulate in subsequent

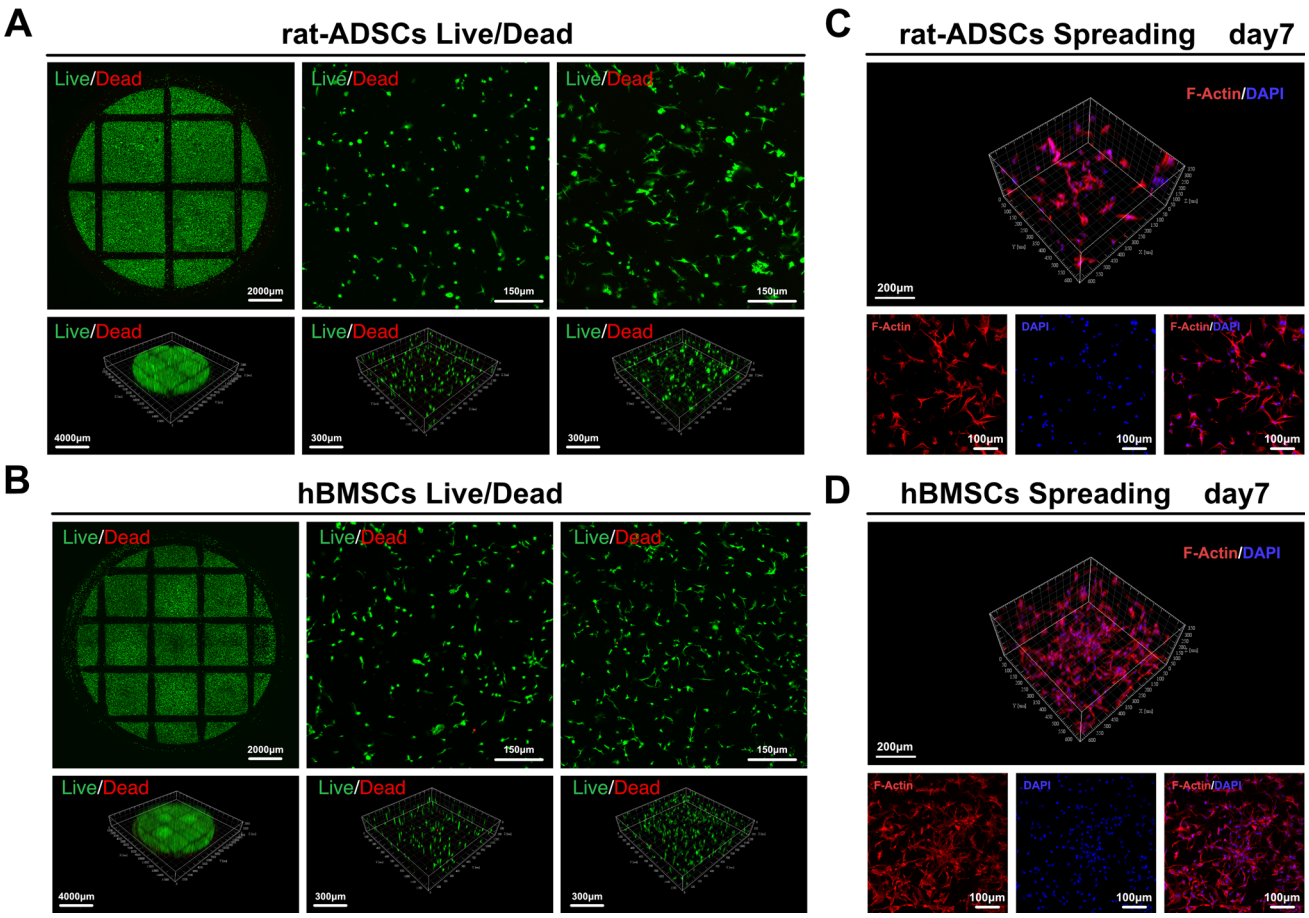

Fig. 2 Cell viability and spreading capability in 3D culturing. **a** Live/Dead of rat ADSCs. **b** Live/Dead of hBMSCs. **c** F-actin/DAPI of rat ADSCs. **d** F-actin/DAPI of hBMSCs

characterizations. Therefore, considering these dilemmas, we invent curing rings, on which the prepared bioink is poured and cross-linked. The grabbing handles or supporting feet on it can make it convenient to grab and form more space for material exchanging, and denser grid can maintain the shape of cross-linked GelMA with lower modulus. Here, the bioink is poured on the curing ring and cured by timing blue light source for 5–10 s.

Step III: Cells collection

After being cultured for several days, the samples can be posttreated for cell examination and analysis. Two situations exist in 3D samples posttreatment. For one thing, hydrogel in some samples do not have to be degraded, and cells can be simply treated such as staining cell products. The stained samples can be directly observed under optical microscopy or confocal fluorescent microscopy. For another, in some biological testing items, such as flow cytometry (FCM), polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA), cells in the 3D samples have to be separated with hydrogel. Thus, we designed GelMA lysis solution, with which the cured GelMA can be easily degraded without cell damage.

Step IV: Biological testing

With the cells collected from Step III, researchers can carry out a series of biological testing items such as microscopy observation, FCM, ELISA, and PCR. According to these testing and analysis results, researchers can conclude the effect of 3D cell culture in vitro and subsequently estimate the effect of different stimulating factors in relevant biomedical applications.

To display this facile workflow more clearly, we captured the illustration video in the Supplementary Information, Video S1.

Examples

Experimental methods

To examine the feasibility of the proposed 3D cell culture protocol, we cultured different kinds of cells including human umbilical vein endothelial cells (HUVECs), rat adipose-

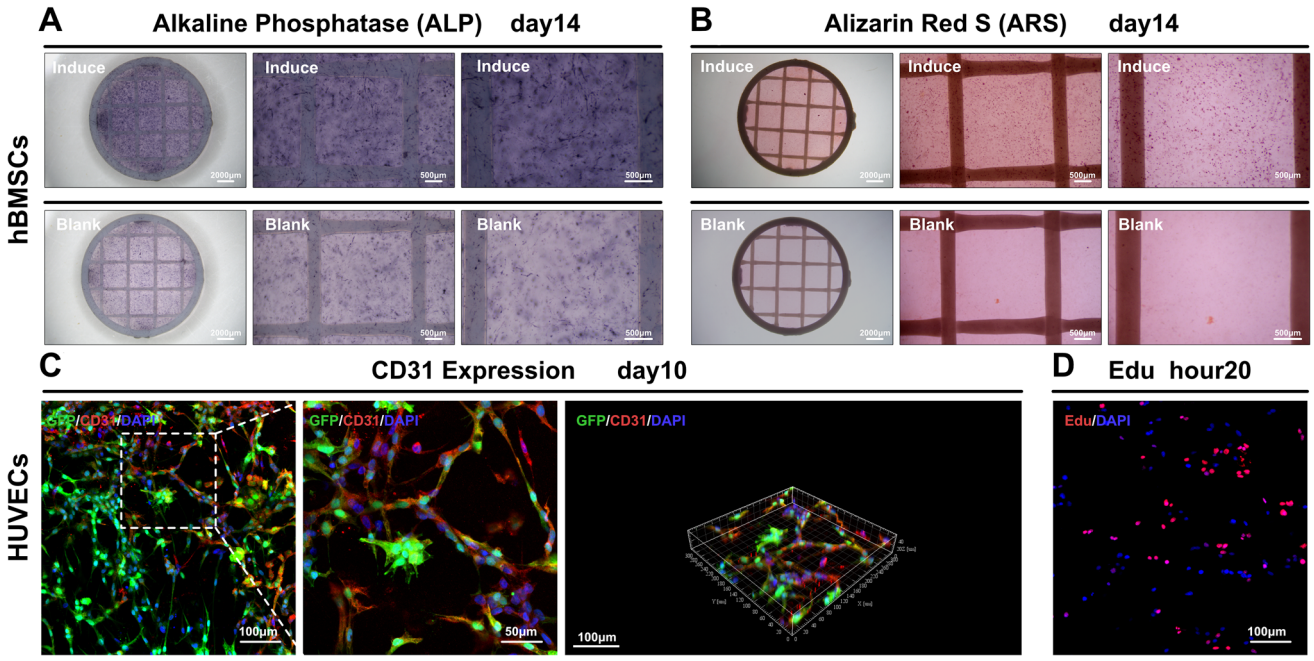

Fig. 3 Cell differentiation in 3D culture. **a** ALP expression of hBMSCs. **b** ARS staining of hBMSCs. **c** CD31 expression of HUVECs. **d** Proliferation of HUVECs

derived stem cells (rat ADSCs), and human bone mesenchymal stem cells (hBMSCs) in this way and characterized their viability, spreading capability, and differentiation. Detailed types of GelMA, curing rings, and blue light source are introduced in Supplementary Information, Tables S1, S2 and Figures S1, S2.

GelMA prepolymer solution was prepared with 5% (w/v) EFL-GM-60, and 0.5% (w/v) LAP. HUVECs, rat ADSCs, and hBMSCs were, respectively, mixed into it and cured on EFL-SCR-2D-24-2. HUVECs and rat ADSCs were cultured in complete medium, and hBMSCs were cultured in osteogenesis induction medium (complete medium + 0.1 mM dexamethasone + 10 mM β-glycerol phosphate disodium salt hydrate + 50 μg/ml L-ascorbic acid). The cells were observed with confocal fluorescence microscopy or optical microscopy.

Experimental results

As shown in Fig. 2a, b, the encapsulated rat ADSCs and hBMSCs show high viability both on the first day and the seventh day of culture. From the general images of the curing ring, we can find that the cells are uniformly distributed in the 3D space inside the cross-linked GelMA. Moreover, as shown in Fig. 2c, d, the encapsulated cells spread in the 3D environment after seven-day culture. This result demonstrates that cells encapsulated in GelMA on 3D curing ring can keep high viability and spreading capability in the proposed 3D culturing protocol.

Moreover, the osteogenesis differentiation of hBMSCs and vascularization differentiation of HUVECs were examined. As shown in Fig. 3a, inducted hBMSCs displayed high alkaline phosphatase (ALP) expression compared with the blank group after 14-day culture. And calcium nodules stained by Alizarin Red S (ARS) in Fig. 3b also demonstrate that the encapsulated hBMSCs realized osteogenesis differentiation. Furthermore, Fig. 3c shows that the encapsulated HUVECs showed high expression of platelet endothelial cell adhesion molecule-1 (CD31) on cell membrane, and Fig. 3d shows obvious cell proliferation.

Discussion

In this technical note, a brand-new 3D cell culture protocol based on photocurable hydrogels is proposed. We designed a series of relevant products, namely GelMA hydrogel, curing ring, timing blue light source and GelMA lysis solution, which can be selected to match this workflow. It is worthy to be mentioned that cell culture on the surface of hydrogel [14–16] is another common culturing style, which is known as 2D culture on scaffolds or 2.5D culture [17]. To illustrate this, we also carried out a series of cell experiments described in Supplementary Information, Additional Discussion, and captured the workflow video displayed in Supplementary Information, Video S1.

In the future, every step in this strategy can be further developed. For example, we can further build 3D ECM based

on other kinds of basic hydrogels, such as gelatin, alginate, and hyaluronic acid. Furthermore, the basic material of cure ring could be changed into functional material, such as conductive material, high-intensity material or materials mixed with biological factors, and nanoparticles in order to add extra stimulations to the encapsulated cells. In conclusion, we believe that this brand-new concept can lead the trend in the field of cell culture and relevant biomedical applications.

Acknowledgements This work was supported by the National Nature Science Foundation of China (Nos. U1909218, 81827804).

Author contributions YH conceived the study; MX and YZ contributed to methodology and investigation; MX helped in writing original draft; all the authors contributed to writing and editing; YH contributed to funding acquisition; QG provided resources; YH supervised the study

Compliance with ethical standards

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.

References

1. Zhu Z, Liu J-D, Liu C, Wu X, Li Q, Chen S, Zhao X, Weitz DA (2020) Microfluidics-assisted assembly of injectable photonic hydrogels toward reflective cooling. Small 16(9):1903939
2. Jensen C, Teng Y (2020) Is it time to start transitioning from 2D to 3D cell culture? Front Mol Biosci 7:33
3. De Leon SE, Pupovac A, McArthur SL (2020) Three-dimensional (3D) cell culture monitoring: opportunities and challenges for impedance spectroscopy. Biotechnol Bioeng 117(4):1230–1240
4. Zhao X, Liu S, Yildirimer L, Zhao H, Ding R, Wang H, Cui W, Weitz D (2016) Injectable stem cell-laden photocrosslinkable microspheres fabricated using microfluidics for rapid generation of osteogenic tissue constructs. Adv FuncT Mater 26(17):2809–2819
5. Xie M, Gao Q, Zhao H, Nie J, Fu Z, Wang H, Chen L, Shao L, Fu J, Chen Z, He Y (2019) Electro-assisted bioprinting of low-concentration GelMA microdroplets. Small 15(4):1804216
6. Xie M, Gao Q, Qiu J, Fu J, Chen Z, He Y (2020) 3D biofabrication of microfiber-laden minispheroids: a facile 3D cell co-culturing system. Biomater Sci 8(1):109–117
7. Xie M, Gao Q, Fu J, Chen Z, He Y (2020) Bioprinting of novel 3D tumor array chip for drug screening. Bio-Des Manuf 3(3):175–188
8. Shao L, Gao Q, Xie C, Fu J, Xiang M, Liu Z, Xiang L, He Y (2020) Sacrificial microgel-laden bioink-enabled 3D bioprinting of mesoscale pore networks. Bio-Des Manuf 3(1):30–39
9. Nie J, Gao Q, Wang Y, Zeng J, Zhao H, Sun Y, Shen J, Ramezani H, Fu Z, Liu Z, Xiang M, Fu J, Zhao P, Chen W, He Y (2018) Vessel-on-a-chip with hydrogel-based microfluidics. Small 14(45):1802368
10. Xie M, Yu K, Sun Y, Shao L, Nie J, Gao Q, Qiu J, Fu J, Chen Z, He Y (2019) Protocols of 3D bioprinting of gelatin methacryloyl hydrogel based bioinks. JoVE 154:e60545
11. Yue K, Trujillo-de Santiago G, Alvarez MM, Tamayol A, Annabi N, Khademhosseini A (2015) Synthesis, properties, and biomedical applications of gelatin methacryloyl (GelMA) hydrogels. Biomaterials 73:254–271
12. Yoon HJ, Shin SR, Cha JM, Lee S-H, Kim J-H, Do JT, Song H, Bae H (2016) Cold water fish gelatin methacryloyl hydrogel for tissue engineering application. PLoS ONE 11(10):e0163902
13. Yuan S, Kang Y, Jing N, Miao S, Jianzhong F, Huiming W, Yong H (2020) Modeling the printability of photocuring and strength adjustable hydrogel bioink during projection based 3D bioprinting. Biofabrication. https://doi.org/10.1088/1758-5090/aba413
14. Zhao X, Lang Q, Yildirimer L, Lin ZY, Cui W, Annabi N, Ng KW, Dokmeci MR, Ghaemmaghami AM, Khademhosseini A (2016) Photocrosslinkable gelatin hydrogel for epidermal tissue engineering. Adv Healthc Mater 5(1):108–118
15. Sun X, Lang Q, Zhang H, Cheng L, Zhang Y, Pan G, Zhao X, Yang H, Zhang Y, Santos HA, Cui W (2017) Electrospun photocrosslinkable hydrogel fibrous scaffolds for rapid in vivo vascularized skin flap regeneration. Adv Funct Mater 27(2):1604617
16. Chen H, Guo L, Wicks J, Ling C, Zhao X, Yan Y, Qi J, Cui W, Deng L (2016) Quickly promoting angiogenesis by using a DFO-loaded photo-crosslinked gelatin hydrogel for diabetic skin regeneration. J Mater Chem B 4(21):3770–3781
17. Prestwich GD (2007) Simplifying the extracellular matrix for 3-D cell culture and tissue engineering: a pragmatic approach. J Cell Biochem 101(6):1370–1383