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Abstract	Developing bioinks that are biocompatible and well-suited with the printing process is crucial for the progress of 3D bioprinting tissue engineering field. Decellularized extracellular matrix (dECM) bioink is considered a promising material for bioprinting applications due to its inherent composition. However, the laboratory protocols for obtaining this bioink involve the use of sodium dodecylsulphate (SDS) detergent which is widely known as an agent that causes lysis of the cell membrane. This study reports the fabrication of decellularized matrix gels and the comparison between the morphology and rheological behavior of the extracellular matrix gels treated with different SDS elimination methods. These dECM gels were treated with PBS, acetone or trichloroacetic acid (TCA). Scanning electron microscopy revealed that the PBS washes and the acetone treatment maintained the porous gel structure. The flow curve of the three gels was also studied and determined that the bioinks has a pseudoplastic behavior and that the viscosity of the TCA treated gel is relatively lower (0.08 Pa·s) than the acetone and PBS treated dECM, 0.2 Pa·s 0.3 Pa·s respectively. Even though the dECM gels treated with acetone and TCA encourages the cell survival, the storage modulus of acetone-treated bioink is above the loss modulus indicating that the microstructure is able to resist the shear stress and hold its conformation.

Keywords	Tissue engineering - Bioprinting - dECM bioink - Sodium dodecyl sulfate (SDS)

Residual SDS Reducing Methods in the Process of Decellularization of Muscle Tissue

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Abstract. Developing bioinks that are biocompatible and well-suited with the printing process is crucial for the progress of 3D bioprinting tissue engineering field. Decellularized extracellular matrix (dECM) bioink is considered a promising material for bioprinting applications due to its inherent composition. However, the laboratory protocols for obtaining this bioink involve the use of sodium dodecylsulphate (SDS) detergent which is widely known as an agent that causes lysis of the cell membrane. This study reports the fabrication of decellularized matrix gels and the comparison between the morphology and rheological behavior of the extracellular matrix gels treated with different SDS elimination methods. These dECM gels were treated with PBS, acetone or trichloroacetic acid (TCA). Scanning electron microscopy revealed that the PBS washes and the acetone treatment maintained the porous gel structure. The flow curve of the three gels was also studied and determined that the bioinks has a pseudoplastic behavior and that the viscosity of the TCA treated gel is relatively lower (0.08 Pa·s) than the acetone and PBS treated dECM, 0.2 Pa·s 0.3 Pa·s respectively. Even though the dECM gels treated with acetone and TCA encourages the cell survival, the storage modulus of acetone-treated bioink is above the loss modulus indicating that the microstructure is able to resist the shear stress and hold its conformation.

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Keywords: Tissue engineering · Bioprinting · dECM bioink · Sodium dodecyl sulfate (SDS)

1 Introduction

One of the most emerging techniques in tissue engineering is bioprinting. The bioprinting process consists of the precise deposition of biomaterial and cells layer-by-layer in pre-defined computer-aided designs. The bioprinting methods are classified into three main groups, according to the operating mechanisms: extrusion-, droplet-, and laser-based process. The comparison of bioprinting techniques in terms of the operating principles, the components, as well as the advantages and disadvantages of each one is described elsewhere [1]. Although, recent advances in the printing of skin

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[2, 3], cardiac cells [4, 5], neural tissue [6], cartilage and bone [7, 8], and stem cell derived tissues [9] have been reported, and in all cases, the suitable bioink features are crucial. Therefore, improving bioinks remains one of the main challenges in tissue engineering for research groups.

One of the most important goals in bioprinting is achieving bioinks whose rheological properties are compatible with the printing method, in addition to maintaining the conditions for cellular function and tissue formation. Encapsulated cells in individual extracellular matrix (ECM) components such as collagen, fibrin, gelatin, hyaluronic acid, etc. are largely adopted for bioink research [10–12]. However, the decellularized extracellular matrix (dECM) based-bioinks promote the cell viability, proliferation, differentiation, migration and morphological changes by growth factors [13].

The dECM bioink is obtained by the elimination of cellular residues through the use of chemical, physical and enzymatic methods. Sodium dodecyl sulfate (SDS) is a detergent commonly used in the protocols for obtaining dECM [14, 15]. The SDS solubilizes the cell and nuclear membrane, plus it efficiently removes the cell content of dense tissues. The impact of SDS on the tissue decellularization process is explained elsewhere [16]. Likewise, the effect of SDS on cell behavior has recently been reported [17]. Because the effect of SDS on cellular and ECM integrity is well known, it is necessary to implement new methods to minimize the remnant of this detergent in the dECM.

Once the dECM is obtained, it is solubilized to the desired concentration, which results in a gel-like substance which must meet several important properties for suitable for bioprinting. The rheological nature of the resulted bioink has a considerable impact on the printability and cellular viability. In consequence, the viscosity of the bioink should be adjustable according to the requirements of the selected printing method.

In the present work, we describe practical methods for reducing the SDS concentration in dECM-based bioink. Reduction of SDS concentration in dECM based-bioinks was enhanced with three different types of treatments. These treatments are the use of acetone (Ac), trichloroacetic acid (TCA), and phosphate buffer solution (PBS). Final SDS concentration, rheological properties and cell viability of fabricated dECM bioinks were also determined and compared.

2 Materials and Methods

2.1 dECM Bioink Preparation

A PBS 10x saline solution was prepared, dissolving 80 g of sodium chloride (NaCl, J. T. Baker), 2 g of potassium chloride (KCl, J.T. Baker), 14.4 g of sodium phosphate dibasic (Na_2HPO_4, J.T. Baker), and 2.4 g potassium phosphate monobasic (KH_2PO_4, Sigma-Aldrich) in distilled water to a final volume of 1 L. To obtain a PBS 1x solution, 100 ml of stock mix was diluted in 900 ml of distilled water. The mixture used to eliminate the cellular content was prepared with 10 g of SDS (Research Products International, IL, USA) dissolved in PBS 1x to a final volume of 1 L.

A porcine muscular tissue sample, obtained from a local meat supplier, was weighted and cut into pieces (up to 8 mm^3). The adipose tissue was removed to avoid

its interference with the detergents. The decellularization of skeletal muscle and vascular tissues was conducted according to the protocol described by Pati *et al.*, 2014 [15] with modification. Briefly, the tissue was stirred in 1% SDS solution (400 ml) for 48 h followed by a treatment with 1% Triton X-100 solution for 30 min. The SDS solution was replaced by the new one at 24 h. Then, the tissue slurry was treated with Tris–HCL solution (pH 7.4) for 4 h followed by 3 washes with PBS solution for 24 h.

2.2 Treatments for the Removal of SDS and Residual Quantification

The samples were weighed and divided in three equal parts named dECM-Ac, dECM-PBS, and dECM-TCA, according to the applied treatment. dECM-Ac sample was shaken vigorously in acetone for 10 min in an ice bath. The sample was centrifuged at 6000 rpm for 5 min. The supernatant was discarded, and the precipitated was transferred to a 35 mm petri dish and dried for 12 h followed by PBS for 24 h. dECM-TCA sample was mixed with 33 ml of trichloroacetic acid (Sigma-Aldrich) (60% in PBS) at room temperature and it was incubated through the night. The sample was centrifugated at 6000 rpm for 5 min. Subsequently, 200 ml of acetone were added for 20 min. Finally, it was washed in PBS for 24 h. The dECM-PBS sample was treated with three consecutive washes of 200 ml of PBS 1x for 24 h each.

The three samples were shaken for 2 h in 250 ml with vigorous agitation of the following: peracetic acid solution (1%), ethanol (4%), and deionized water. After this moment, the samples were manipulated under sterile conditions inside a biosafety cabinet class 2. All samples were rinsed with a sterile PBS solution for 24 h.

The samples were lyophilized for 12 h. Afterward, the samples were weighed and 100 ml of HCl 0.01M were added for each gram in the sample, as well as pepsin. The mixture was maintained with agitation for 48 h and afterwards treated with a NaOH 0.1M solution to obtaining a final pH 7.4. The samples were filtered with a nylon mesh of 100 μm (Corning). Subsequently, 6 μL of solubilized gel were mixed with 594 μl of a methylene blue solution (Fisher Scientific), along with 1200 μl of chloroform (J. T. Baker). The absorbance of the samples was measured at 650 nm (Thermo Scientific NanoDrop 2000) and quantified by referring to a standard curve made in advance with SDS.

2.3 Morphology Characterization

The morphology of dECM samples was characterized using a Scanning Electron Microscope SEM (JEOL6010 PLUS/LA) operating at 5.0 kV. The dECM sample was instantly frozen in liquid nitrogen for 2 min and lyophilized for 24 h before being cut into small pieces with a sharp blade. The samples were deposited onto an aluminum holder before SEM observation.

2.4 Rheological Characterization

A rotational rheometer (AR-2000, TA Instruments) with cone geometry (60 mm diameter, 500 μm gap) and a solvent trap was used to evaluate rheological properties such as viscosity (η) and storage modulus (G′) of the dECM-based bioinks. A shear rate

sweep was done in a range of 0.01 s^{-1} to 100 s^{-1} and the apparent viscosity was registered. In addition, it was conducted a temperature sweeps from 8 °C to 36 °C, by simulating a transition from the refrigerator to the incubator. The storage module of the material was measured in function of time. Every measurement was taken in triplicate under the same temperature conditions.

2.5 Cell Culture on Gels

Cell cultures were performed by using breast cancer cell line MDA-MB-231, which expresses the green fluorescent protein (GFP) in its nucleus. The treated gels were mixed with the cell line at a concentration of 2×10^6 cells/ml. A 96-well plate was seeded with 75 µL of the mixture and allowed to incubate at 37 °C for one hour and a 2 ml of culture media was added. The plate was kept in an incubator at 37 °C and 5% CO_2. Changes of culture medium were made every 3 days for a week and then every 2 days since the cell population had increased.

An inverted microscope, U-HGLGPS lighting system, a cubic filter set for GFP and the Metamorf Advance software (IX-83, Olympus, Japan) were used to monitor cell culture growing. The lamp was set to a light intensity of 6% and the images were captured with an exposure time of 100 ms, with a separation between images on the z-axis of 5 µm. The range where the cells were located was searched manually and between 40 and 150 photographs were taken from each well, during days 4, 8 and 17 after starting the cell culture. The files were saved in .TIFF format and the contrast and lighting were adjusted using the ImageJ tool. To show representative results of the cell population throughout the gel, a sum of the images was performed. For this, the Z-Projection tool was used with a maximum intensity projection, which shows the maximum value of the pixels parallel to the z-axis.

3 Results and Discussion

During the decellularization process changes in color and consistency of the muscular tissue were observed. The dECM product is shown in Fig. 1, and the changes are due to the detergent's action which removes blood, lipids and cell content. Once the decellularization process and the methodology implementation to eliminate the SDS were finished, the PBS washed sample was filtered, and the acetone and TCA treated samples were dried. The PBS-treated product did not show any changes after the washes. On the other hand, the acetone precipitated pregel was reduced in size, and it adopted a yellowish color and, once rehydrated, it recovered its initial appearance.

The SDS concentration in the solubilized dECM-treated bioink was measured by adding chloroform and methylene blue to the sample, and the absorbance of the organic phase was measured and compared with the absorbances of the known concentrations. As seen in Table 1, the PBS-treated gel showed a higher SDS concentration, whereas the acetone-treated gel showed the lowest concentration.

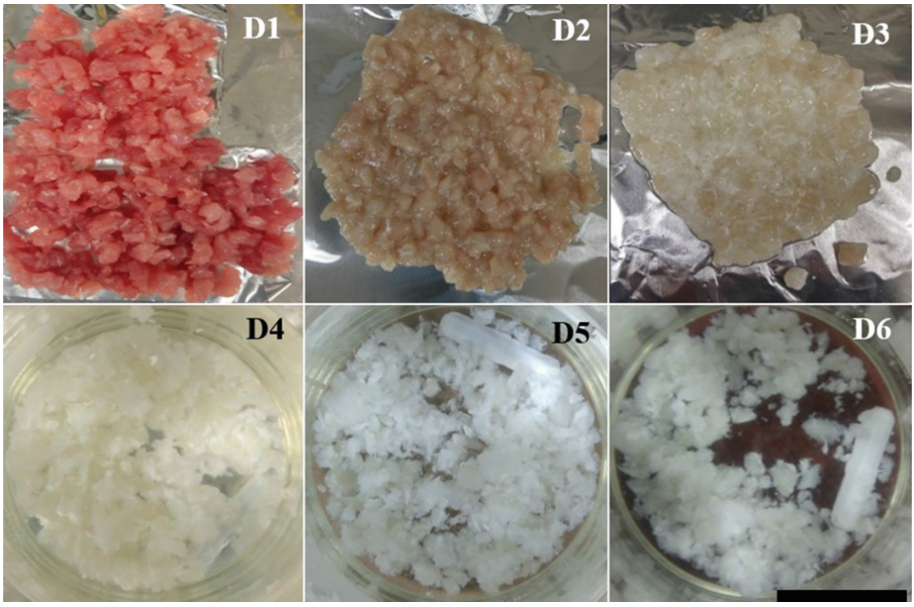

Fig. 1. Images of a native and decellularized muscular tissue sample. The changes were monitored for 6 days. The 1% SDS solution was replaced by the fresh one at 24 h. Scale bar = 45 mm

Table 1. Residual concentration of SDS in dECM inks after treatments

Treatment	Absorbance	Concentration, % (m/V) 10^{-3}
Acetone	0.206[a]	3
Trichloroacetic acid	0.240[b]	5
Phosphate buffer solution	0.325	9

[a]Acetone-treated gel presented an absorbance lower than the LOD
[b]TCA-treated gels showed values below the LOQ, previously calculated.

SEM images are shown in Fig. 2 and demonstrate porous morphology in the PBS-dECM and Ac-dECM while TCA-dECM shows a morphology with no pores. Intermolecular coalescence of unfolding protein is derived from the exposure of the non-polar surface produced by TCA [18] which causes the proteins to form macromolecular aggregates, and avoids the formation of pores.

The results of the flow curves of three different dECM bioinks (Fig. 3) suggest that the dECM gels are suitable for bioprinting techniques compatible with low-viscosity bioinks, as jetting-based printing (3.5–12 mPa·s), and droplet-based printing (<15 mPa·s). However, the dECM bioink treated with TCA was ineffective to form stable porous morphology due to its relatively low viscosity, whereas the PBS-dECM and Ac-dECM with relatively higher viscosity formed a stable porous morphology.

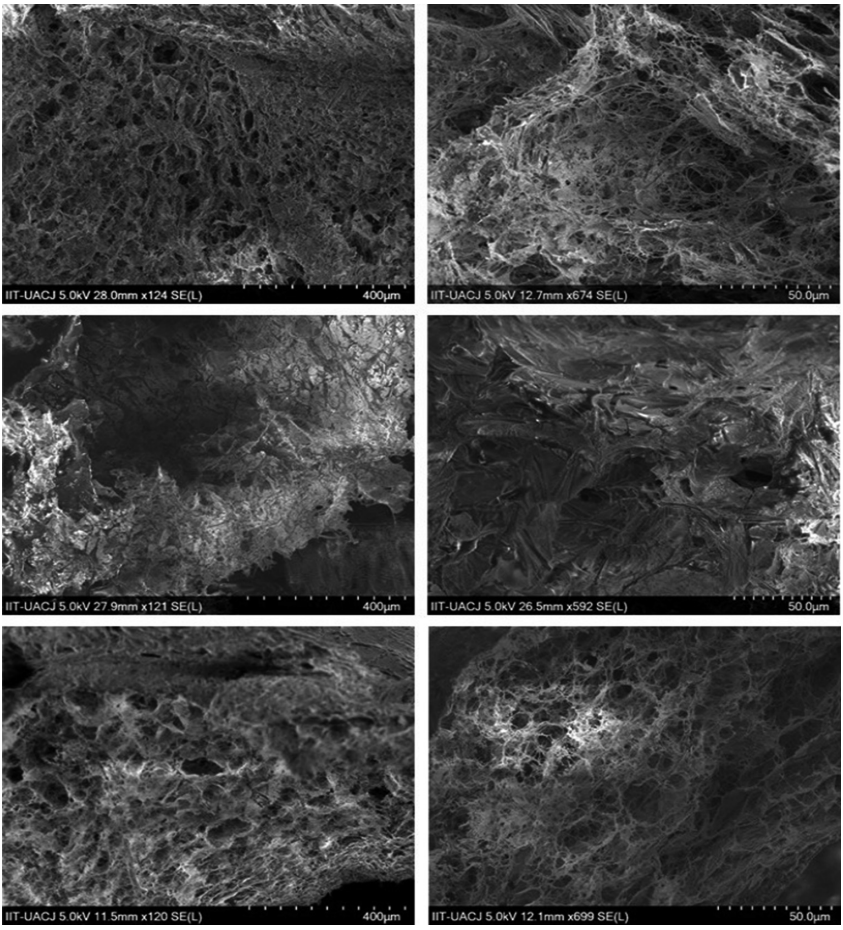

Fig. 2. Representative SEM micrographs of PBS-dECM (top), TCA-dECM (middle), and Ac-dECM (bottom)

The inherent mechanical properties of the biomaterials have a crucial role in the printability of the material, resolution of the impression and cell viability. The information obtained from the flow curve, and the temperature step indicate a modification in the structure of the proteins. This increases the viscosity of the solution at low shear rates and accelerates the gelation process. As shown in Fig. 4, the loss modulus of the bioinks dipped below the storage modulus, due to the thermal gelation properties of dECM. However, the storage modulus is always above the loss modulus in Ac-dECM. This result indicates that although the bioink may flow above 36 °C, the porous microstructure is able to resist the shear stress and hold its conformation.

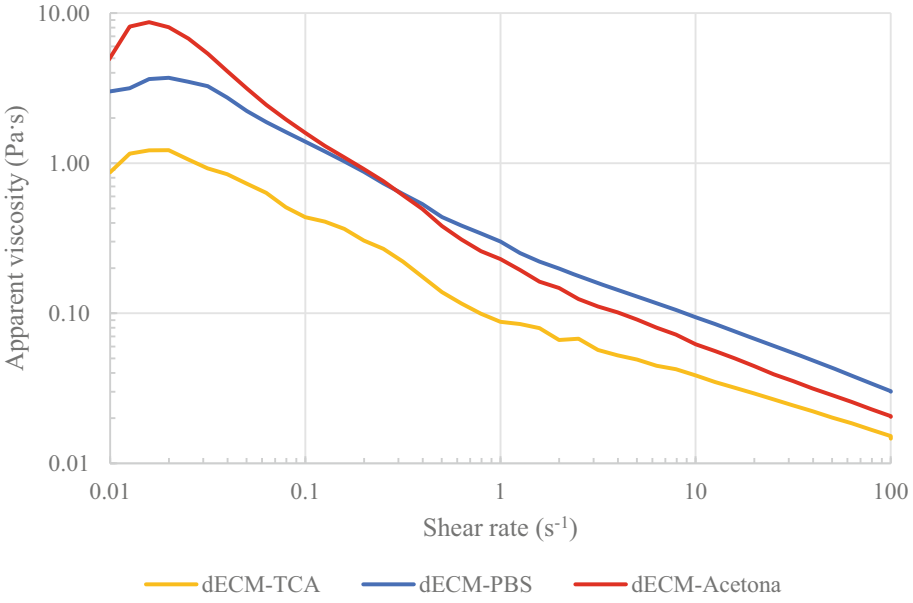

Fig. 3. Flow curves of three different dECM bioinks

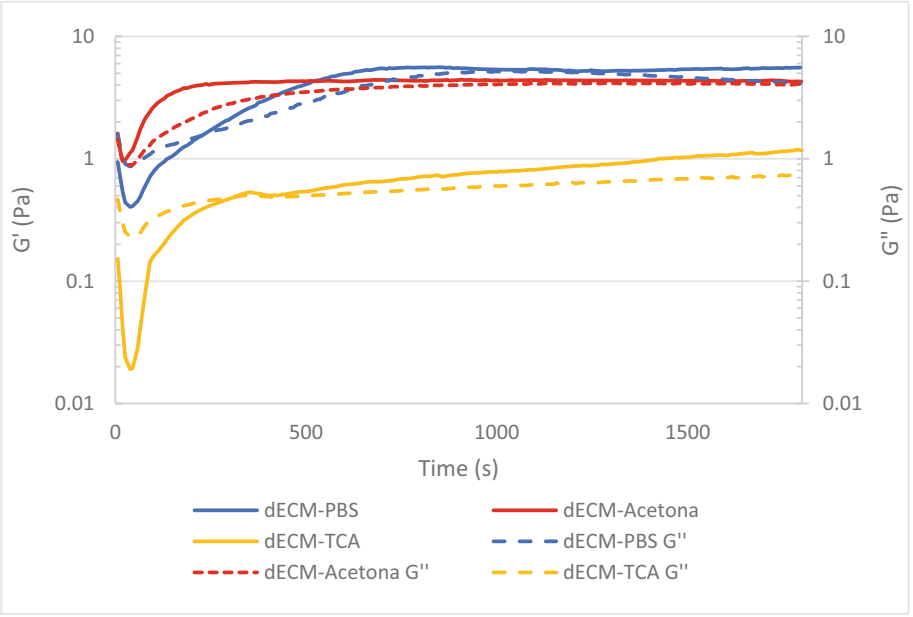

Fig. 4. Storage and loss modulus after a temperature step

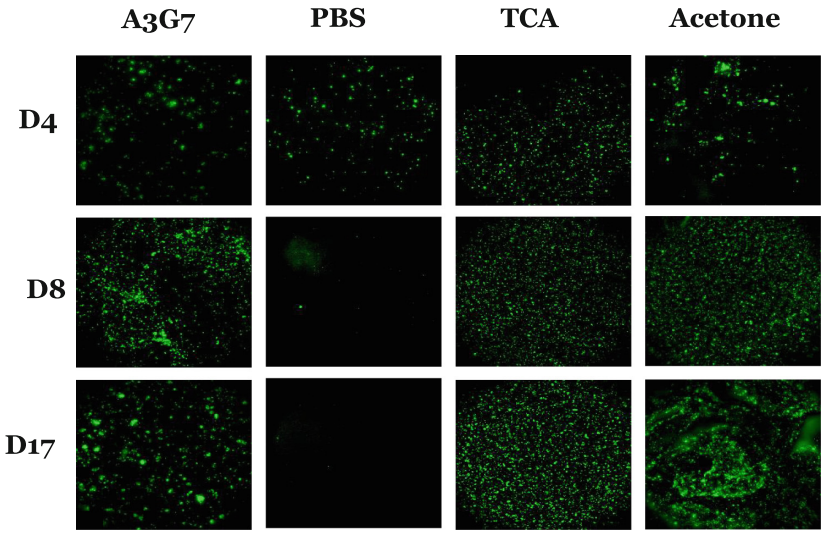

Fig. 5. Cell cultures in extracellular matrix gels from day 4 to day 17. Column from right to left: Ac-dECM, TCA-dECM, PBS-dECM and A3G7. The images were captured at 10× magnification

As shown in Fig. 5, a gel of 3% alginate and 7% gelatin (A3G7) was used as control on cell cultures. The development of tumor spheroids was observed, as previously reported [19]. The cell population increased during the 17 days of cell culture in the control gel, and in the gels treated with acetone and TCA. On the other hand, the culture performed in gels washed with PBS had negative effects on the cell population, since it practically eliminated the number of green spots on day 17. What indicates the presence of a cytotoxic element in the gel that was not removed by the PBS washes and that correlates with the highest concentration of SDS measured in the colorimetric test. When comparing the dECM gels treated with acetone and TCA with the control, a greater number of green points was observed on day 17, suggesting that the dECM gels treated with acetone and TCA encourages the development of the cell population. It was also found that the cells proliferated throughout the dECM gel while the cells in the control gel were kept in groups, forming tumor spheroids.

4 Conclusion

This study has suggested a viable strategy to remove SDS from dECM from muscular tissue. The final SDS concentration was below detectability levels, as confirmed after cell culture. Although modified, the rheological are suitable for bioprinting with different printing systems. This finding confirms the usefulness of acetone as a treatment to reduce the SDS concentration in the dECM. Moreover, the method proposed has a clear advantage over the treatment of removing SDS by several washes with PBS as reported in previous literature.

References

1. Noh, S., Myung, N., Park, M., Kim, S., Zhang, S.-U., Kang, H.-W.: 3D bioprinting for tissue engineering. In: Clinical Regenerative Medicine in Urology, pp. 105–123. Springer, Singapore (2018)
2. Yan, W.-C., et al.: 3D bioprinting of skin tissue: from pre-processing to final product evaluation. Adv. Drug Deliv. Rev. **132**, 270–295 (2018)
3. Augustine, R.: Skin bioprinting: a novel approach for creating artificial skin from synthetic and natural building blocks. Prog. Biomater. **7**(2), 77–92 (2018)
4. Cheung, D.Y.C., Duan, B., Butcher, J.T.: Bioprinting of cardiac tissues. In: Essentials 3D Biofabrication Translation, pp. 351–370 (2015)
5. Kuss, M., Duan, B.: 3D bioprinting for cardiovascular tissue engineering. In: Rapid Prototyping in Cardiac Disease, pp. 167–182. Springer, Cham (2017)
6. Gu, Q., Tomaskovic-Crook, E., Wallace, G.G., Crook, J.M.: Engineering Human Neural Tissue by 3D Bioprinting, pp. 129–138. Humana Press, New York (2018)
7. Chen, C., et al.: Research trends in biomimetic medical materials for tissue engineering: 3D bioprinting, surface modification, nano/micro-technology and clinical aspects in tissue engineering of cartilage and bone. Biomater. Res. **20**(1), 10 (2016)
8. Park, S.-H., Jung, C.S., Min, B.-H.: Advances in three-dimensional bioprinting for hard tissue engineering. Tissue Eng. Regen. Med. **13**(6), 622–635 (2016)
9. Tasnim, N., et al.: 3D bioprinting stem cell derived tissues. Cell. Mol. Bioeng. **11**(4), 219–240 (2018)
10. Parak, A., Pradeep, P., du Toit, L.C., Kumar, P., Choonara, Y.E., Pillay, V.: Functionalizing bioinks for 3D bioprinting applications. Drug Discov. Today **24**(1), 198–205 (2019)
11. Hospodiuk, M., Dey, M., Sosnoski, D., Ozbolat, I.T.: The bioink: a comprehensive review on bioprintable materials. Biotechnol. Adv. **35**, 217–239 (2017)
12. Jang, J., Park, J.Y., Gao, G., Cho, D.-W.: Biomaterials-based 3D cell printing for next-generation therapeutics and diagnostics. Biomaterials **156**, 88–106 (2018)
13. Nam, S.Y., Park, S.-H.: ECM Based Bioink for Tissue Mimetic 3D Bioprinting, pp. 335–353. Springer, Singapore (2018)
14. Deepak, C., Han Win, T., Tianyi, W., May Win, N.: Organ-derived decellularized extracellular matrix: a game changer for bioink manufacturing? Trends Biotechnol. **36**(8), 787–805 (2018)
15. Pati, F., et al.: Printing three-dimensional tissue analogues with decellularized extracellular matrix bioink. Nat. Commun. **5**(1), 3935 (2014)
16. Crapo, P.M., Gilbert, T.W., Badylak, S.F.: An overview of tissue and whole organ decellularization processes. Biomaterials **32**, 3233–3243 (2011)
17. White, L.J., et al.: The impact of detergents on the tissue decellularization process: a ToF-SIMS study HHS. Acta Biomater. **50**, 207–219 (2017)
18. Rajalingam, D., Loftis, C., Xu, J.J., Kumar, T.K.S.: Trichloroacetic acid-induced protein precipitation involves the reversible association of a stable partially structured intermediate. Protein Sci. **18**(5), 980 (2009)
19. Jiang, T., et al.: Directing the self-assembly of tumour spheroids by bioprinting cellular heterogeneous models within alginate/gelatin hydrogels. Sci. Rep. **7**(1), 4575 (2017)

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