Peptide-Based Bioink Development for Custom-made Bioprinter with Specialized Nozzle Design

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Abstract*—Recently, bioprinting is an emerging technology that is widely used in regenerative medicine and tissue engineering. To obtain a fully functional tissue, all the properties and functions of the desired tissue must be considered and adapted to the material and cell line for the bioprinting procedure. Therefore, the bioink should not only be highly biocompatible to accommodate cells, but also should be suitable for the bioprinting procedure. Hydrogels are ideal candidates for bioinks to create artificial tissue scaffolds due to their reproducibility, tunability, printability, biocompatibility, similarity to the natural extracellular matrix, as well as their optimal framework in which they support cell viability and proliferation. The absence of biological cues on synthetic hydrogels requires the necessity of additional modification to provide efficient cell adhesion, and differentiation. Herein, it is aimed to design a universal biofunctionalized bioink that can be used in every laboratory by optimizing the printability of the bioinks and also to design custom-made nozzle with various diameter. First, YIGSR, which is bioactive laminin derived peptide, used for modification of KLDLKLDLKLDL self-assembled peptide (SAP) with N-terminal addition and polyethylene glycol diacrylate (PEGDA) with chemically modification and produced as hydrogel bioinks and viscosity was measured. Then, human bone marrow stromal cells (hBMSC) cells were encapsulated in hydrogels and cell viability analysis was carried out by MTT and Live/Dead analysis for 7 days. Custom-made nozzles with various diameter were produced using glass capillary tubes and flow was analyzed with COMSOL according to the hydrogel viscosity properties and the shape of the nozzles. The results of this study will help the laboratories to produce their own bioinks, the improvement of extrusion-based 3D bioprinting

Keywords — Tissue engineering, peptide, hydrogel, bioprinting, bioink, hBMSC

I. INTRODUCTION

Three-dimensional (3D), bioprinting is used in producing tissue scaffolds, tissues, and organs by bioinks that involve hydrogels, bioactive molecules, and cells to obtain specified constructs by shaping the structure layer by layer [1]. In order to obtain a fully functional tissue, all the properties and the functions of the tissue to be obtained must be considered and it should be adapted to the bioprinting procedure and the cell line. The ideal bioink formulation should meet specific material requirements such as printability, degradability, functionality and also biological requirements such as biocompatibility and bioactivity. When the material properties are considered, printability might be considered as the most important parameter, and viscosity has a great effect on printability. Viscosity affects both cell encapsulation efficiency and printing quality. When the requirements are considered from a biological perspective, the bioink and its degradation products should not contain materials that induce an inflammatory response when implanted, at this point, besides using biocompatible materials, using patients' cells to create a bioink would highly decrease the rejection risk by the body.

There are plenty of hydrogels used in bioinks such as alginate, collagen, gelatin, gelatin methacryloyl, and polyethylene glycol diacrylate (PEGDA). These polymers can be used alone in bioink formulation or they can be conjugated with bioactive molecules such as proteins, growth factors and peptides to increase bioactivity to drive cellular behaviors such as cell adhesion, migration and differentiation. PEGDA hydrogels are often preferred in 3D bioprinting. However, PEGDA is resistive to protein adsorption and does not have active molecules to provide specific cell attachment. Therefore, conjugation of PEGDA with different bioactive molecules may increase biological activity. Hydrogels consist of self-assembled peptides (SAP) are started to be used widely in tissue engineering applications regarding of their ability of mimicking both functional and structural properties of extracellular matrix (ECM). Molecular self-assembly is formed by the interactions of the unorganized molecules arranging among themselves, however, it must be supported with a bioactive epitope to increase the bioactivity. When the accessibility of bioinks is considered, they are not extensively produced and the companies that produce bioinks are limited worldwide. Also, these commercially available products are high-cost and maintaining conditions are difficult while delivering them. When these difficulties are considered, the need for developing biofunctional bioinks in laboratories has been emerged. Herein, KLDLKLDLKLDL (K:Lys, L:Leu, D:Asp) self-assembled peptide (SAP) hydrogel and PEGDA hydrogel are individually combined with YIGSR (Y:Tyr, I:Ile, G:Gly, S:Ser, R:Arg) peptide which is laminin derived peptide to increase the cell viability, and functionality. Using YIGSR functionalized PEGDA and SAP hydrogels as bioink will provide the cells ECM mimetic environment and make the physicochemical properties of bioinks great options for mimicking a tissue [2].

Not only the mechanical and biological properties of the bioinks affect the cell viability but bioprinting nozzles also have an effect on cell viability and bioprintibility. Nozzles are the devices that are placed to the tip of the 3D bioprinter, they provide substituent flow to outer space through them. Nozzles can be in different shapes, sizes, diameters and they can be made of different materials and cell viability of a 3D bioprinted scaffold is affected by these parameters. Importantly, it is known

978-1-6654-5432-2/22/$31.00 ©2022 IEEE

that the conical nozzle provides higher cell viability compared to the tubular nozzle due to the proportionally increasing shear stress on the cells [3].

In this study, we aimed to design a universal biofunctionalized bioink that can be used in every laboratory by optimizing the printability of the bioinks and also design custom-made nozzle with various diameter. First, YIGSR, which is bioactive laminin derived peptide, used for modification of KLDLKLDLKLDL SAP with N-terminal addition and PEGDA with chemically modification and produced as hydrogel bioinks and viscosity was measured. Then, human bone marrow stromal cells (hBMSC) cells were encapsulated in hydrogels and cell viability analysis was carried out by MTT and live/Dead analysis for 7 days. Custom-made nozzles with various diameter which are suitable to the extrusion-based 3D bioprinter previously designed in our laboratory, were produced using glass capillary tubes and flow was analyzed with COMSOL according to the hydrogel viscosity properties and the shape of the nozzles. We believe that the results of this study will help the laboratories to produce their own bioinks, the improvement of extrusion-based 3D bioprinting.

II. Material and Method

A. Self-Assembled Peptide Hydrogel-based Bioink Production

Solid phase peptide synthesis was performed according to the previous study [4]. KLDLKLDLKLDL-NH_2 and KLDLKLDLKLDLYIGSR-NH_2 (KLD-YIGSR) SAP were synthesized on Rink Amide NovaGel resin (0.62 mmol/g). 200 mg of resin was left in 3 ml Dimethylformamide (DMF) solution to swell for 30 minutes and then washed with DMF (2x3 ml). Fmoc-protected amino acid derivatives (2 eq), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium Hexafluorophosphate (HBTU; 1.95eq) and *N, N*-diisopropylethylamine (DIEA; 3 eq) were dissolved in DMF (3 ml) and added to the resin. The mixture was stirred in an orbital shaker for 4-6 hours at 30°C. 20% piperidine/dimethylformamide (DMF) solution were used for Fmoc deprotection for 20 min after each coupling step. A small amount of resin was separated and the Kaiser test was applied to check for the presence of unreacted amines. If the test results were positive, the resin was washed with DMF (5x3 ml) and the reactions were repeated until a negative result was obtained. When the test results were negative, the resin was first washed with DMF (5x3 ml), then kept in 20% piperidine/DMF for 30 minutes to remove the Fmoc-protecting group. The following amino acids were produced using the same method until all amino acids in the sequence were added to the peptide sequence. The resin was then left in trifuoroacetic acid (TFA) (95% (v/v)), triisopropylsilane (TIPS) (2.5% (v/v)), deionized water (2.5% (v/v)) solution for 2 hours to extract the peptide sequence from the resin. The solution was poured into cold ether and the product was precipitated at 4500 rpm for 10 minutes at 4°C for 3 repetitions. Then the suspension was centrifuged, the supernatant was removed, the remaining ether was removed by vacuum evaporator and the last solid part was lyophilized by freeze drying method. For the preparation of SAP based bioinks, 4% KLD and KLD-YIGSR hydrogels were prepared by dissolving 4 mg of peptide in 100 µl of hydrogel. The self-assembly of the peptides was initiated by adding an equal volume of cell growth medium (pH 7.4). Buffer solutions were used to trigger self-assembly, which varied according to the nature of the measurements. The buffer used here is Dulbecco's Modified Eagle Medium (DMEM) cell culture medium supplemented with 25 mM HEPES buffer, free of fetal bovine serum (FBS). The solution was transferred into a sterile syringe and used in the printing process.

B. Peptide Conjugated PEGDA Hydrogel-Based Bioink Production

YIGSR peptide was synthesized according to the method explained in the previous section. For PEGDA-peptide conjugation, two buffers were prepared by dissolving 563.76mg sodium phosphate dibasic ($NaH_2PO_4 \cdot H_2O$) in 10ml distilled water and 276mg sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$) in 10ml distilled water. 0.265ml monobasic and 4.735ml dibasic buffer were mixed with 5ml distilled water. PEGDA was dissolved in 100 mM sodium phosphate buffer (pH 8.0). After PEGDA:Peptide solution was prepared at 1:1.1 molar ratio, it was allowed to react for 24 hours by stirring in a dark place. The PEGDA-YIGSR solution was then transferred to a dialysis tube and dialyzed in distilled water for 24 hours, by changing the medium on a regular basis. After freezing at -80°C, the solution was lyophilized to obtain a powder [5]. 10% PEGDA and PEGDA-YIGSR hydrogels were prepared by dissolving 5 mg 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiopheno (Irgacure 2959) in 1000 µl of phosphate buffered saline (PBS) with the help of a sonicator and then mixed it with 100 mg PEGDA and PEGDA-YIGSR powders. The resulting solution was passed through a 0.22µm filter for sterilization. Then, it was shaped by performing the cross-linking process with the UV (Ultraviolet) device (365nm) inside the custom-made bioprinter with 3.5cm and 5cm distance for 180 sec and 240 sec, respectively (Figure 1A)

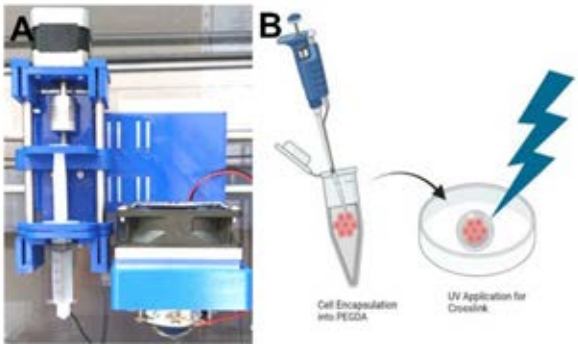

Figure. 1. A) UV curing system in the bioprinter designed in our laboratory B) UV application to the bioink for crosslinking after cell encapsulation into the PEGDA based bioinks.

C. Cell Encapsulation into Hydrogels

Human bone marrow derived mesenchymal stem cells (hMSCs) (HMSC-AD-500, CLS cell lines Service, Lot #102, Eppelheim, Germany) were used in the experiments and cultivated in the medium consists of DMEM, 10% FBS, and 100 U/ml penicillin. After the cells were seeded in standard polystyrene cell dishes in this medium, they were incubated at 5% CO_2 and 37°C, and the medium was changed every two

days. Passaging was accomplished when the cell density reached 90%. The passage process was performed with 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA) solution at the appropriate passage rate. $5x10^6$ cells/mL were encapsulated in PEGDA, PEGDA-YIGSR, KLD and KLD-YIGSR solutions. Cell encapsulated PEGDA was exposed UV for 180 sec from 3cm and 5 cm distance to optimize cell viability (Figure 1B). Then, cell encapsulated PEGDA-YIGSR exposed UV for 180 sec from 3 cm distance which is optimized previously. KLD and KLD-YIGSR hydrogels were assembled with the addition growth medium (pH 7.4) in equal volume. The hydrogel scaffolds were cultured in the presence of DMEM medium in a 5% CO_2 incubator at 37°C for 24 hours. The medium change was made regularly and cells were cultured in the indicated medium for up to one week.

D. MTT Analysis

For evaluating the cell viability of the cells encapsulated into the hydrogel based bioinks, MTT (Vybrant ® MTT Cell Proliferation Assay Kit (Invitrogen) were performed on days 1, 4, and 7. For this analysis, 3-(4,5-dimethylazol-2-yl)-2,5-diphenoltetrazol bromide) MTT was dissolved in 5 mg/mL PBS solution and filtered using a 0.22 µm syringe. On the 1st, 4th and 7th days, the medium was removed from each well. Then 10 µl of MTT and 90 µl of DMEM were added into wells. After the plates were incubated at 37 ºC for 2 hours, the solution in the wells was removed. After adding dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, Missouri, USA) as much as MTT solution (100 µl) to each well, it was left in a shaker for 15 minutes in order to homogenize the solution better, and then optical density was measured at 570 nm using a microplate reader (Synergy™ HTX- BioTek, Winooski, VT, USA).

E. Live/Dead Analysis

On the 7th day, the Live/Dead test was applied in addition to the MTT test. For this, 4 µl of Calcein, 20 µl of Eth were mixed in 2 ml of sterile PBS in the dark. After removing the medium from the cell encapsulated gels in the wells, 100 µl of the prepared solution was added to the gels. Then it was placed in the incubator at 37ºC for 45 minutes. At the end of 45 minutes, the solutions were removed from the gels, washed twice with sterile PBS, and the PBS was removed from the wells and the gels were imaged with the help of a microscope.

F. Nozzle Design and Flow Simulation

Nozzles are produced using glass capillary tubes which are made of glass which were provided kindly by Dr. Mustafa Şen (Izmir Katip Celebi University, Turkey). Firstly, capillary is placed to the PC-10 micropulling machine (Narishige, Japan), the heat was applied to the middle of the capillary tube, then the middle point is elongated by applying a force by the two ends. Finally, two nozzles with conical shapes are obtained. Since the diameters of the nozzles are unknown, diameters are determined under a microscope by assigning scale bar. Until reaching the required diameters, nozzles are grinded using a microgrinder (EG-401, Narishige, Japan). Eventually 100µm, 200µm, 300µm and 400µm diameter nozzles are obtained. For the simulation, the STL file of nozzles with different diameters were created by SolidWorks according to the microscope images.

For the simulation, viscosity of bioinks was measured by discovery hybrid rheometer-2 (HR2, TA Instruments, New Castle, DE). The viscosity values of 1 ml of samples were recorded with some certain parameters such as the gap of 0.5 cm, angular frequency of 0.1-10 rad/s with changing shear stress values. The bioink flow in custom-made nozzles during bioprinting was simulated COMSOL Multiphysics Simulation 5.4 to find essential parameters in terms of velocity and pressure.

III. Results And Discussion

MTT assay and Live/Dead test results are shown in Figure 2A and 2B, respectively. Survival of the cells that have been exposure to UV light differ depending on the different parameters of the light source such as UV exposure time and distance between the hydrogel and UV source [6, 7]. Herein, to optimize the cell viability depending on UV exposure time and the distance for crosslinking of PEGDA based hydrogels, with 3.5cm and 5cm distance for 180 sec and 240 sec, respectively and it is observed that the duration spent for crosslinking is increased proportionally with the distance of the UV light to the sample to obtain similar hydrogel structure. 5cm distance UV application on PEGDA hydrogels resulted in lower cell viabilities and determined that the killer effect of UV light applied from 3.5cm distance on the cells encapsulated into the PEGDA is lower compared to the 5cm distance. Therefore, the experiments on PEGDA-YIGSR were continued with 3.5cm distance by allowing the cells in the samples to be cultivated in a healthy way [6, 8].

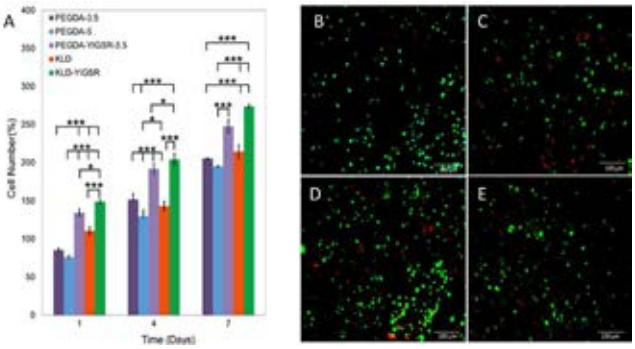

Figure. 2. A) Cell number of hBMSCs that were encapsulated into hydrogel bioinks. [Significant differences were determined by one-way ANOVA Newman-Keuls multiple comparison test (*p\0.05, **p\0.01, ***p\0.001). Live/Dead test results of B) KLD C) PEGDA-YIGSR D) KLD-YIGSR E) PEGDA hydrogels bioinks. Scale bar represents 100 µm.

ECM has an important effect on cell adhesion and cell growth [9]. Since, YIGSR peptide encourages cell proliferation, it has been widely used in functionalization of biomaterials [10]. By conjugating YIGSR peptide, which has the ability to mimic ECM domain that triggers the cell behavior in a positive way, to PEGDA, higher cell attachment and proliferation was obtained compared to the PEGDA using the same UV the parameters (180 sec, 3.5cm). Also, KLD SAP hydrogel without the need of a light source, were performed with encapsulating cells. To support the effect of YIGSR peptide on cell proliferation,

experiments carried on with both KLD and KLD-YIGSR hydrogels. Cell viability and live/dead results confirmed that that KLD-YIGSR hydrogel has supported more cell attachment and proliferation with higher viable cells compared to KLD. Therefore, the KLD-YIGSR has greater supportive effect on cell proliferation compared to KLD sequence in accordance with the literature [11]. As a result, although PEGDA-YIGSR and KLD-YIGSR have the same peptide epitope and the same cell density, UV exposure affected the process negatively. However, PEGDA-YIGSR is more controllable in bioprinting compared to the SAP hydrogel, since self-assembling of KLD and KLD-YIGSR is temperature depended.

The nozzles were designed in SolidWorks after imaging under the microscope to use in simulation (Figure 3). Viscosity values which are illustrated in Figure 4 demonstrated that viscosity was increased by the addition YIGSR to both PEGDA and KLD bioinks and each value is used for the simulation. The solution and nozzle tip interaction, flow rate and viscoelasticity, were simulated using the Multiphysics simulation program COMSOL 5.4 to decide on suitable solution and nozzle tip (Figure 5). Although the velocity on walls and output decreases with increasing diameter of nozzle tips in the same solution, they did not change significantly in a different solution, which had approximate viscosity. However, apart from velocity, pressure changed dramatically in both different solution and different diameter. It increased about eight times when comparing 100µm KLD and KLD-YIGSR.

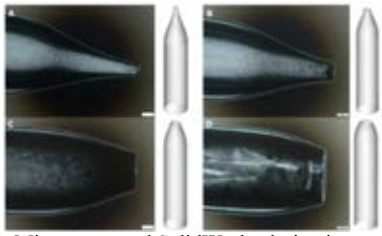

Figure. 3. Microscope and SolidWorks design images of nozzles with A) 100µm, B) 200µm, C) 300µm, D) 400µm diameters. Scale bar represents 200µm.

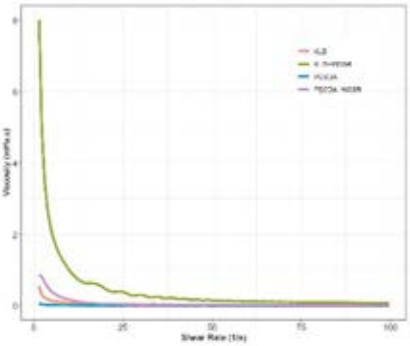

Figure. 4. The viscosity values of bioinks measured by a rheometer at room temperature

ACKNOWLEDGEMENT

This project is supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK) within the scope of 2209B Research Projects.

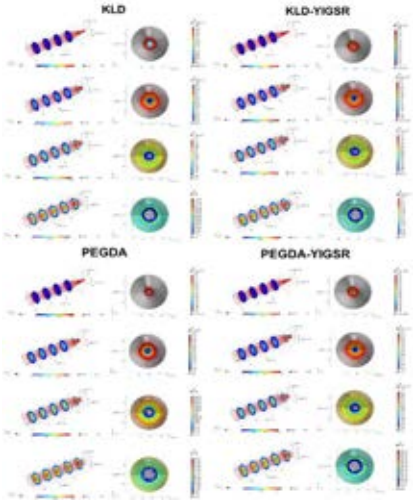

Figure. 5. Comparison of the velocity and pressure simulation with different bioink and nozzle with changing diameters solution obtained by a COMSOL.

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