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Assessing the Bioprintability of Self-Assembling Peptide Bioinks in Terms of Structure Fidelity and Cell Viability

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Abstract— **Organ donor shortage as well as an increasing demand for personalized medicine have opened up new avenues in tissue engineering. As 3D bioprinting may provide promising solutions, bioinks of different compositions are being developed to serve bioprinting needs. As for the development of suitable bioinks, certain challenges and limitations still exist including: The use of inorganic, unnatural or undefined natural materials, UV and chemical crosslinking for gelation, and fidelity of 3D structures. Self-assembling peptides boast an advantage of resembling human-like materials and activating instantaneous gelation. In this paper, ultrashort peptides are used for 3D bioprinting. The printed scaffolds are analyzed for structure fidelity, cell viability, and proliferation. The results are compared with commercial Biogelx™ peptide bioinks as a benchmark. Our custom-designed robotic 3D bioprinter is used and compared with the commercial Inkredible+ bioprinter. Our results prove the bioprintability of self-assembling peptide IK6 (Ac-ILVAGK-NH_2) with enhanced cell viability and structure fidelity. Importantly, our results clearly demonstrate the potential use of Self-Assembling peptides as superior bioinks for various tissue engineering applications.**

Keywords—3D bioprinting, peptide, Biogelx™, biomaterials, bioinks, tissue engineering

I. Introduction

Scarcity of donors for organ transplantation has increased the need for engineered tissues. Numerous challenges remain in developing tissues and organs appropriate for medical translation. Nevertheless, initial successes in simple tissue engineering were experimented[1]. Three-dimensional (3D) bioprinting technology has facilitated the fabrication of complex tissue structures. The construct is produced by dispensing biomaterial or cell-laden hydrogels in a layer-by-layer formation. The printing of these hydrogels via micro-channels demands a tedious process with accurate stacking of layers to fabricate tissue[2].

In bioprinting, the technology is as significant as the bioinks. Existing bioprinting techniques are inkjet bioprinting, stereolithography, laser-assisted bioprinting, and extrusion-based bioprinting [3, 4]. Each method has its specific advantages and disadvantages. Inkjet printers, which use the drop-on-demand printing method, are considered to have the fastest and most cost-effective printing process [3]. However, highly viscous hydrogels cannot be printed in inkjet bioprinters, as the risk of the nozzle clogging is great. On the other hand, the laser-assisted bioprinting technique relies on the theory of laser-induced forward transfer. Its key feature is the ability to print with a broader range of viscous hydrogel bioinks. Also, it can maintain the viability of a high number of cells. Nonetheless, this bioprinting method is considered costly, and it is relatively slow compared to the inkjet bioprinters. Furthermore, the stereolithography approach is commonly used in tissue engineering as it can print with high resolution. But the risk of cytotoxicity due to the fabrication process reduces cell viability[3]. Extrusion-based bioprinting is the most commonly used bioprinting method because it tolerates highly viscous hydrogels while printing with a significant number of cells[3, 4].

Despite the existence of various hydrogel bioinks, most require a crosslinking process which dramatically affects cell viability. The process includes exposure to UV light or solidifying the hydrogel via chemical crosslinking to transform the bioink into a more stable scaffold. A number of natural bioinks were experimented to test their applicability for 3D bioprinting[5-8]. Collagen and alginate-based natural bioinks have been developed, yet they are not fully compatible for human use which makes them unsuitable for human transplants.

Ultrashort self-assembling peptides have been evolved and investigated as biomaterials for *in vitro* cell culture, implantable scaffolds and regenerative medical applications. They have demonstrated their potential as a bioink for bioprinting and biofabrication[9-12]. Moreover, ultrashort peptide bioinks show adequate mechanical strength, firmness, and shape fidelity. A major advantage is the synthesis of the peptide from essential amino acids which are biocompatible and non-immunogenic[12]. Due to their viscous properties, peptide bioinks require extrusion-based printing, where viscous peptide aggregates laden with cells are extruded from a microfluidic nozzle[13-15]. The conventional printing mechanism used for extrusion-based bioprinting is the Cartesian linear system. Much like commercial 3D printers, motors are positioned along the x-y-z axes and move linearly with rods and pulleys. The ink is pumped either through a screw-driven or a pneumatic-driven system[4].

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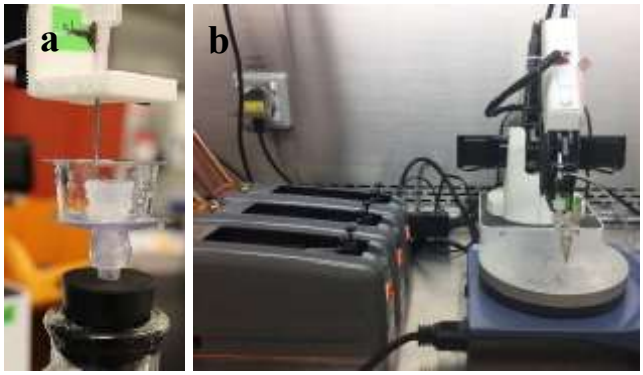

Figure 1 Our in-house developed robotic 3D bioprinting system with (a) a vacuum unit, printing a peptide hydrogel and (b) with a heater prepared for printing cell-laden structures.

Herein, we report a preliminary study on ultrashort peptide-based hydrogels using a custom-developed 3D bioprinting system. The study aims to assess and compare the bioprinting performance of different peptide bioinks, i.e. an ultrashort hexameric peptide-based hydrogel, IK6 (Ac-ILVAGK-NH_2),with a commercially available peptide bioink, Biogelx™. Experiments have been conducted to analyze the structure fidelity, cell viability and cell proliferation of each 3D printed construct with different peptide concentrations. Moreover, the comparison of the bioinks was performed with two different 3D bioprinting systems, our in-house developed robotic 3D bioprinter and the commercially available 3D bioprinter, Inkredible+. The Inkredible+ is a linear 3D bioprinter which uses a pneumatically driven system for extrusion. On the other hand, our in-house developed robotic 3D bioprinter uses a 4 degree-of-freedom robotic arm for added precision and flexibility[16]. The extrusion mechanism relies on screw-driven microfluidic pumps. The results of the experiments are presented with analysis of the data.

II. Materials and Method

Five comparative experiments were conducted for each of the two investigated peptides, IK6 (Ac-ILVAGK-NH_2) and Biogelx™. IK6 was custom synthesized by Bachem in a purity of >95% via HPLC. Biogelx™-INK-GFOGER was purchased from Biogelx™. Factors studied included shape fidelity, cell viability, cell proliferation and working parameters of our 3D bioprinter. All experiments were independently repeated three times.

Several sequences of ultrashort peptides [9-12] were compared for 3D bioprinting. IK6 was chosen due to its quick gelation, high fidelity and low viscosity which makes it a good candidate for printing with screw-driven microfluidic pumps.

A. *Structure Fidelity Assessment*

An experiment was setup to compare the structure fidelity of both bioinks, IK6 and Biogelx™. Our bioprinting system, described earlier [16], was used for both peptide bioinks with a vacuum unit [17, 18] to print identical 3D structures. Then the results were compared.

The system was setup for bioprinting as shown in Figure 1a. The vacuum was aligned with a 0.4µm PET membrane[14]. A 3D hollow cylinder was designed with dimensions of 10x10x200mm and loaded onto the printing software. Two microfluidic syringe pumps were used for the experiment. Pump 1 was loaded with 5x PBS to enhance gelation. Pump 2 was loaded with the peptide hydrogel being tested.

An amount of 40 mg of Biogelx™ peptide was diluted in 1 mL MilliQ water, which is the suggested concentration for bioprinting according to the company's protocol. After sonication, the pre-gel solution was loaded in a syringe and fixed on to pump. Biogelx™ pre-gel solution was loaded in Pump 1 and attached to the custom-made two inlet nozzle[16]. Multiple flow rates were tested to provide optimal gelation. The pumping flow rates for the Biogelx™ solution and 5x PBS were set to be 65 µL/min and 25 µL/min, respectively. The vacuum pump was activated and a 3D hollow cylindrical structure was printed. The printing was stopped once the peptide material was completely used.

Likewise, an amount of 20 mg of IK6 peptide solution was mixed with 1 mL MilliQ water. The solution was sonicated and placed in an incubator at 37ºC for 30 minutes.

The same printer and pump settings were used to print with IK6 peptide bioink. The optimal flow rates for IK6 and 5x PBS were 70 µL/min and 20 µL/min, respectively. Once the peptide bioink was completely used, the printed samples were removed from the vacuum system and placed in petri dishes. Both samples used 2mL of peptide bioink to print 38 layers of peptide. The height of both structures was measured and compared.

B. *Cell Viability and Proliferation*

Cell culture. NIH 3T3 cells were provided by the Cell Therapy Center, University of Jordan, Amman. Cells were maintained in fresh medium comprising of Dulbecco's modified Eagle's medium (DMEM) (Gibco), supplemented with 10% bovine calf serum (HyClone), and 100U/ml penicillin and 100µg/mL streptomycin (Gibco) in a CO_2 incubator at 37°C and with 6.5% CO_2. The cells were split using trypsin (0.125%; Gibco) at approximately 80% confluence. The culture media was changed every 2–3 days. Cells at passages 3-5 were used for the bioprinting experiments.

IK6 peptide was weight at two concentrations of 15 mg/mL and 20 mg/mL. The peptide powders were sterilized for 30 minutes under UV light. Then, each peptide powder was dissolved in 1 mL of MilliQ water using a shaker.

The bioprinting system was prepared for printing as shown in Figure 1b. The heater was set to 37ºC to ensure a suitable temperature environment for the cells once printed. The .gcode file for printing was set for a filled cube of 4 layers and dimensions of 10x10x1.5 mm.

The two pumps were loaded for printing. Pump 1 was loaded with the pre-gel IK6 peptide with concentration of 15 mg/mL and set to a flow rate of 60 µL/min. Pump 2 was loaded with $6x10^6$ 3T3 cells in media and set to a flow rate of 20 µL/min. The same procedure was repeated for a concentration of 20 mg/mL of IK6. In this case, the flow

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Figure 2 Inkredible+ 3D Bioprinter from Cellix® [19], used to print the Biogelx™ peptide hydrogel[20]

rates of Pump 1 and Pump 2 were set to 65 µL/min and 25 µL/min respectively.

Following the Biogelx™ company protocol[20], an amount of 40 mg of Biogelx™-INK- GFOGER powder was weighed out for a 1 mL solution. The pre-gel solution was prepared using 700 µL of deionized water. It was then incubated at 4ºC overnight to allow nanofiber formation to occur. 225 µL of media was then added to initiate crosslinking. The formulation was incubated for 2 hours at 37ºC until it reached a suitable viscosity for printing. NIH 3T3 cells ($3x10^6$ cells/mL) in 75 µL of media were added to complete the solution.

The Biogelx™ peptide hydrogel was loaded in the commercial ink cartridge of the Inkredible+ Bioprinter [Figure 2]. The same printing file was loaded of a 4 layer cube. The pressure was varied between 11-15 kPa until the extrusion was consistent and a solid cube of 4 layers was printed. Results were recorded.

The same procedure was repeated for a concentration of 20 mg/mL of Biogelx™ to compare the effects of lower concentrations of peptide. The pressure for this test was set to 15 kPa. The same file was printed and results were recorded.

To confirm the capability of our 3D bioprinter to print with commercial bioinks, Biogelx™ peptide was printed with our system and the results were compared with those obtained when printing with Biogelx™ peptide in the Inkredible+. Biogelx™ peptide was measured out in two concentrations of 20 mg/mL and 40 mg/mL. They were sterilized for 30 minutes under UV light. Then, 1 mL of MilliQ water was added and dissolved using a shaker. After sonication, the vials were incubated at 37ºC for 2 hours. The IK6 peptide was not tested with the Inkredible+ Bioprinter as pressure control was not ideal to form a consistent hydrogel at the set concentration.

Our bioprinting system was setup with the same protocol. Pump 1 was loaded with Biogelx™ peptide 20 mg/mL and set to a flow rate of 65 µL/min. Pump 2 was loaded with $6x10^6$ 3T3 cells in DMEM media. The flow rate was set to 20 µL/min. For 40 mg/mL of peptide, the flow rates of Pump 1 and Pump 2 were set to 60 µL/min and 20 µL/min, respectively.

Post-printing procedure. Immediately after printing, the constructs were placed for 5 minutes in the CO_2 incubator at 37°C and 5% CO_2 in order to improve the gelation of the bioink. After 5 minutes, complete culture medium was added to each construct and then placed in the CO_2 incubator at 37°C and 5% CO_2. The culture media was changed every 2–3 days. The viability of the cells within the constructs was examined using Live/dead staining, a two-color fluorescence assay (LIVE/DEAD Viability/Cytotoxicity Kit; Invitrogen) consisting of calcein as a marker of living cells and ethidium homodimer as a marker of dead cells. Briefly, bioprinted tissues were washed 3 times in PBS and treated with calcein AM (green) and ethidium homodimer-1 (red) at 1:2 ratio in PBS. The samples were then incubated for 20 minutes in the dark at 37°C and 5% CO2. After staining, samples were washed 3 times with 1x PBS. A confocal microscope (Leica SP8) was used for image acquisition.

III. Results & Discussion

In our first set of experiments, the printability of IK6 and Biogelx™ bioinks was compared to confirm the fidelity of the printed constructs. The structure printed with the IK6 peptide solution showed good stability as shown in Figure 3b. The instantaneous gelation properties of the IK6 peptide facilitated smooth and consistent extrusion of the bioink, thus resulting in a finer and more stable construct of 15 mm height. Using Biogelx™ peptide bioink, the 3D printed construct had a height of 9 mm, which is significantly shorter than the IK6 construct [Figure 3a].

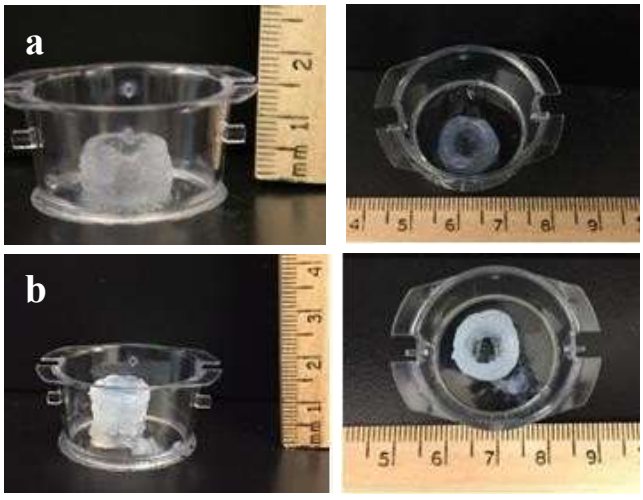

Figure 3 3D printed constructs (a) A 9 mm height construct was printed using Biogelx™ 40 mg/mL with our robotic 3D bioprinter and a 3D printed construct of 15 mm height was printed using 20 mg/mL IK6 with our robotic 3D bioprinter (b)

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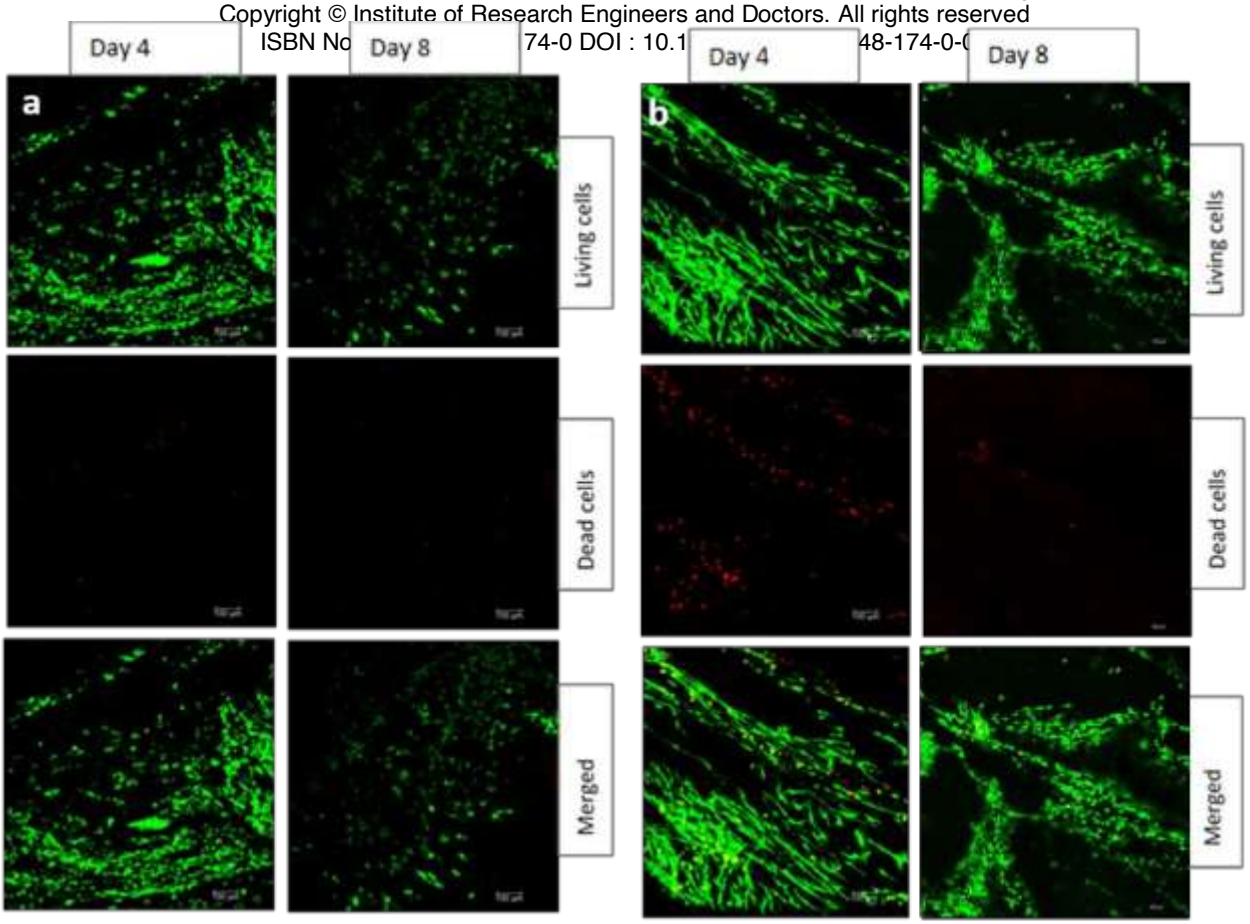

Figure 4 Representative image of bio-printing with our in-house developed robotic 3D bioprinting system using IK6 peptide. (a) 15mg IK6, results of day 4 and 8 post printing. (b) 20mg IK6, results of day 4 and 8 post printing. To assess the viability and morphology of cells, cells were stained with calcein-AM (green; live cells) and ethidium homodimer-1 (red; dead cells). Results from day 4 and 8 show the presence of high percentage of viable cells with stretched morphology (green) and presence of only low number of dead cells (red).

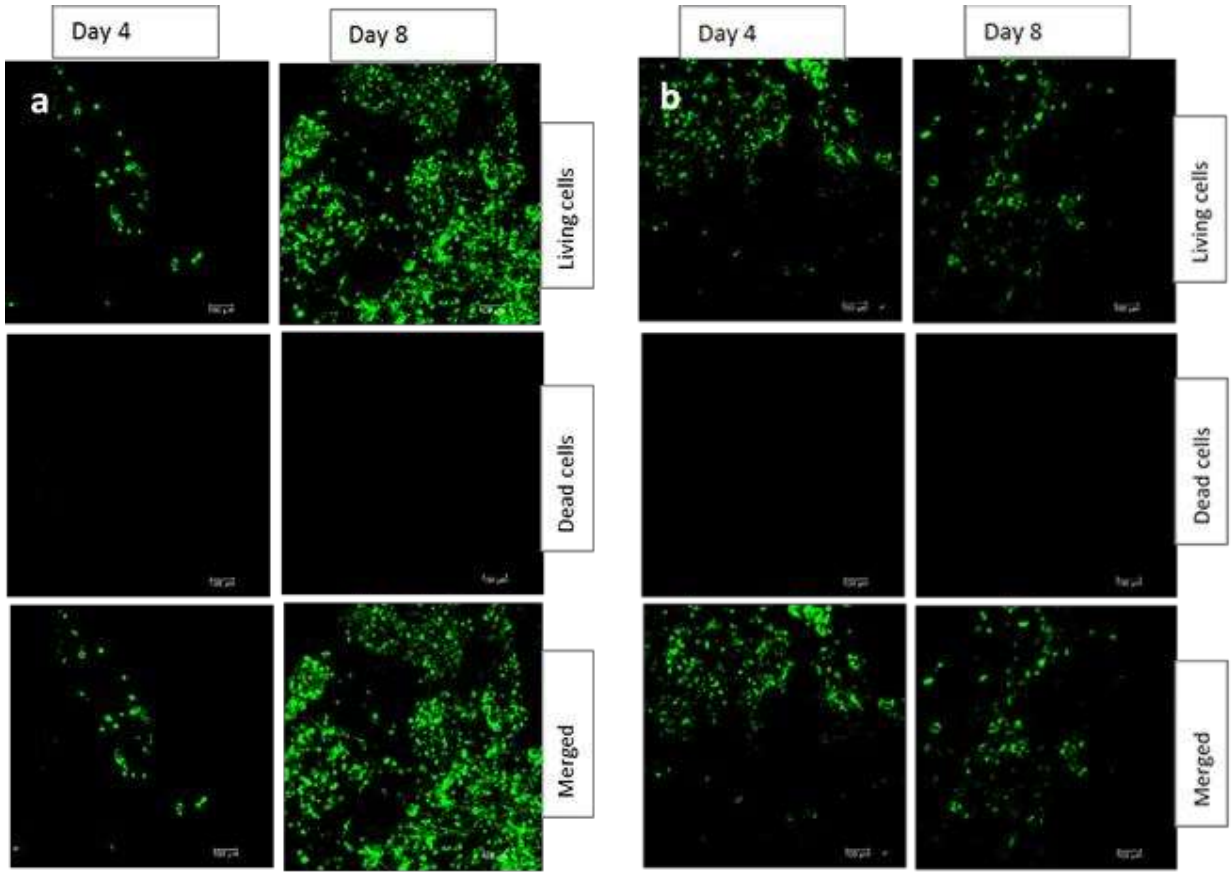

Figure 5 Representative image of bio-printing with Inkredible+ bioprinter system using Biogelx bioink. (a) 20mg Biogelx, results of day 4 and 8 post printing. (b) 40mg Biogelx, results of day 4 and 8 post printing. To assess the viability and morphology of cells, cells were stained with calcein-AM (green; live cells) and ethidium homodimer-1 (red; dead cells). Results from day 4 and 8 clearly show most of the cells possessed an atypical morphology (round shape, green). Indicating the unsuitability of Biogelx bioink, at used concentration, as a bioink for the bioprinting of fibroblasts.

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Figure 6 3D printed samples showing hydrogel constructs including 3T3 cells in media after 7 days using different bioinks. From left to right, IK6 15 mg, IK6 20 mg, Biogelx™ 20 mg, Biogelx™ 40 mg.

To determine the cell viability during bioprinting, we assessed the viability of 3T3 fibroblasts on day 1, day 4, and day 8 after printing. Using our IK6 peptide bioink, the live/dead cell assay showed ≥90% cell viability, which was maintained through days 4 and 8 [Figure 4a-b]. Moreover, fluorescent confocal microscopy images showed that cells attained excellent spreading within the IK6 scaffolds in two concentrations of peptide, 15 mg and 20 mg. The results also showed that the hydrogel promoted the growth and proliferation of bioprinted cells particularly at Day 4. However, a slight decline in cell viability was found in Day 4 when using 20 mg of IK6. This might be due to nutrient and O_2 diffusion limits when using higher concentration of peptide. Nevertheless, the healthy cells in the 20 mg sample proliferated, leading to increased viability reaching ≥90% within day 8 [Figure 4b].

Similarly, using Biogelx™, the cell viability was monitored using live/dead assay. The results showed ≥90% cell viability until day 8 [Figure 5a-b]. It was noted, however, that the cells maintained in round structures and did not show any signs of stretching throughout the period of observation. The number of living cells remained consistent without any signs of proliferation. It was also observed that the 3D cube constructs did not stay intact by Day 7 and showed signs of disintegration into the media [Figure 6]. Both the 20 mg and 40 mg samples showed similar results in terms of cell viability [Figure 5a-b]. The experiment was initially conducted with 40 mg peptide concentration according to the Biogelx™ protocol but was found to clog easily in the nozzle due to high viscosity [Figure 5b]. For this reason, the concentration was reduced to 20 mg [Figure 5a].

To assess whether our custom-designed robotic 3D bioprinting system would be easily compatible with commercial peptides, such as Biogelx™, we compared the printing results from our system with the printing results from the commercial Inkredible+ bioprinter. This experiment was performed under similar conditions while maintaining the same peptide concentration. Similar results were obtained - the cells still appeared to be round without any stretching or signs of proliferation [Figure 7a-b]. This confirms that the observed cell morphology, when using

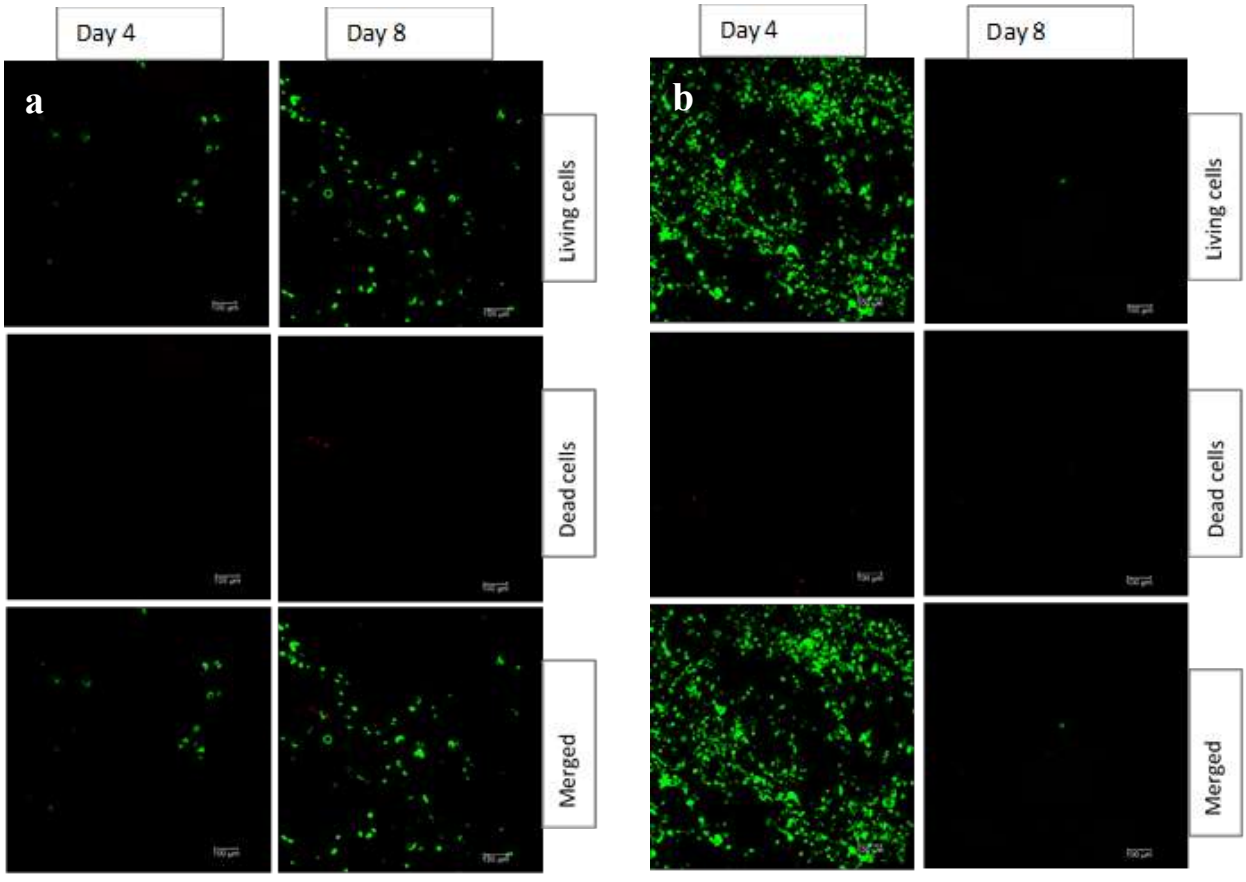

Figure 7 Representative image of bio-printing with our in-house developed robotic 3D bioprinting system using Biogelx bioink. (a) 20mg Biogelx, results of day 4 and 8 post printing. (b) 40mg Biogelx, results of day 4 and 8 post printing. To assess the viability and morphology of cells, cells were stained with calcein-AM (green; live cells) and ethidium homodimer-1 (red; dead cells). Results from day 4 and 8 clearly show most of the cells possessed an atypical morphology (round shape, green). Indicating the unsuitability of Biogelx bioink, at used concentration, as a bioink for the bio-printing of fibroblasts. Scale bar corresponds to 100 µm in size.

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Biogelx™ peptide, was related to the peptide bioink inherent properties rather than the bioprinting system. According to our results, we conclude that our robotic bioprinting system could be suitable for bioprinting using different type of bioinks. Further tests will verify the system's performance with collagen and alginate-based bioinks.

IV. Conclusion

This study offered valuable insights on the performance of the IK6 peptide bioink compared to the commercial Biogelx™ peptide bioink. Our experiments confirmed that IK6 could be used to successfully print 3D structures without negatively impacting the cell viability. Confocal microscopy showed signs of 3T3 cells stretching and proliferating, which was better than expected. Biogelx™ also proved to produce living cells, though images showed round cells without stretching. In terms of structure fidelity, IK6 produced a taller construct of 15 mm as compared to the Biogelx™ construct of 9 mm. The custom-designed 3D robotic bioprinting system was proven to be able to print with Biogelx™ and produce similar results when compared to the Inkredible+ system. The next phase of this study would be to further verify the results obtained through cytoskeletal staining, ABT assays, and broadening the scope of applications using other type of bioinks.

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Author Contributions

C.A.E.H supervised the project and finalized the manuscript. K.K. and Z.K. together with C.A.E.H. designed the study, conducted the 3D bioprinting experiments, and wrote the manuscript. D.A. and F.M. performed cell culturing, live/dead staining, and confocal microscopy.

Conflict of Interest

The authors declare that they do not have any competing interests.

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