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Patient-Derived Hydrogel as a Sacrificial Matrix for Efficient Cell Loading

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Abstract

The developing field of tissue engineering focuses on delivering patient-derived stem cells to the body through the use of degradable biomaterials, such as hydrogels, which are infused into engineered scaffolds. These hydrogels act as templates to support, sustain, and guide cells to the regeneration of new tissue within and beyond the scaffold. In this study, we introduce a completely intraoperative procedure for obtaining a fibrin hydrogel for use in cell therapy and tissue engineering applications. In the past, fibrin hydrogel has been commonly formed by combining fibrinogen protein with animal-derived thrombin. Instead, we have developed an automated, and therefore reproducible, protocol to isolate and form fibrin hydrogel without the use of animal-derived thrombin by using the patient's own peripheral blood. By substituting calcium for animal-derived thrombin, we engineer a completely autologous hydrogel that eliminates the risk of disease transmission, immunogenic response, and FDA regulation. After engineering the fibrin hydrogel, we characterized it as a supportive, sacrificial matrix, and then ultimately confirmed that hybrid hydrogel-PLGA scaffolds increase cell efficiency.

First, cell viability studies confirmed that fibrin gel increases cell loading and retention as compared to the scaffold alone because the hydrogel helps "trap" cells. Then, confocal microscopy images depicted that the hydrogel serves as a supportive network for the cells to survive and grow in. Therefore, utilizing this patient-derived hydrogel as a sacrificial matrix within a scaffold increases cell efficiency, ultimately enhancing tissue regeneration therapies. This method can be effectively implemented to further develop completely intra-operative tissue engineering strategies (CITES) that can be easily translated into the clinic for patient use.

Introduction

Tissue engineering aims to regenerate the patient's own tissue via the use of biodegradable biomaterials and scaffolds with the use of cells and/or signaling molecules *(1)*. Advantages of such regenerative strategies for healing include the fact that they negate the need for replacement surgeries, permanent implants, and decrease the risk of infection or failure. Tissue engineering strategies involve biomaterials, cells, and signaling molecules, either alone or in combination *(1-2)*. The development and discovery of biomaterials as matrices for cell loading and growth have led to great advancements in the field of tissue engineering *(3-6)*.

In order to engineer a tissue in vitro requires, cells are required to inhabit degradable matrices, which will degrade at the same rate as the new tissue growing. Current strategies focus on the use of stem cells, including embryonic stem (ES) cells, bone marrow mesenchymal stem cells (BM-MSCs) and umbilical cord-derived mesenchymal stem cells (UC-MSCs). Adult BM-MSC have been previously utilized to show how a generic marrow cell population can differentiate into an osteogenic lineage, which can then be utilized to augment the repair of bone *(32)*. Mesenchymal stem cells, for instance, can be differentiated into various lineages based on the growth factors and environment that they are cultured in (Fig. 1). In the laboratory setting, cells are cultured in an incubator at 37^{o}C with 5% P_{CO2}. As cells utilize the nutrients from the media, they grow and multiple, eventually leading to confluency, which is the saturation of cells in the plate (Fig. 1B). At this point, the cells are ready for passage and division into two plates so they can continue to grow and multiply. In this study particularly, Human Mesenchymal Stem Cells (HMSCs) were cultured and utilized for testing.

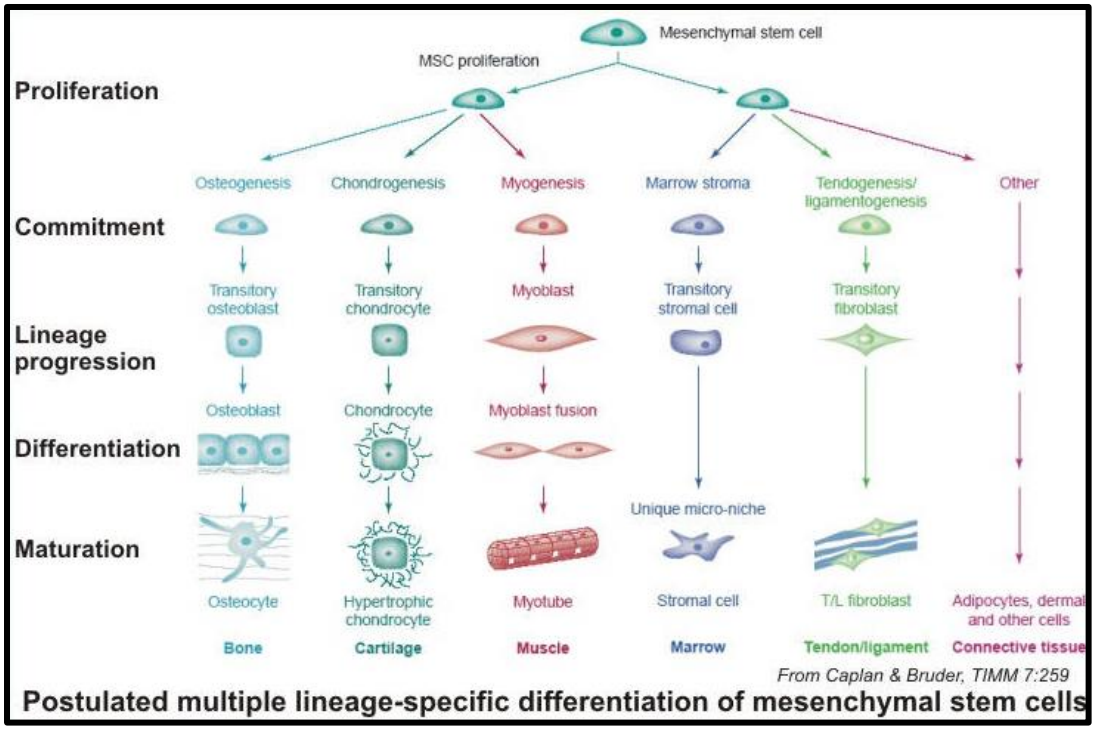

Fig. 1A. Differentiation of mesenchymal stem cells from a single origin into multiple lineages

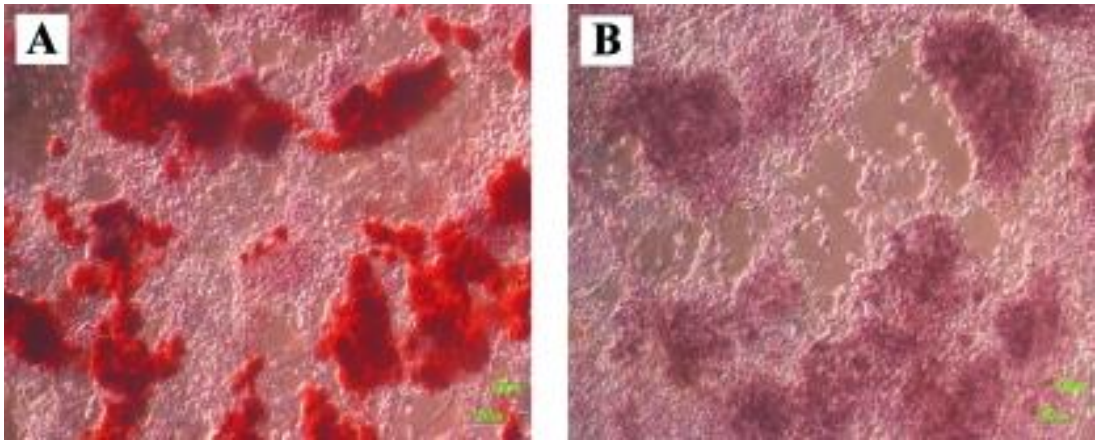

Fig. 1B (A-B). Differentiation of mesenchymal stem cells from a single origin into the osteogenic lineage shown by (A) alizarin red-stained mineral accumulation compared with (B) control (field of view 1100 × 950 µm) *(32)*

In order to create an environment for the patient-derived or engineered stem cells to thrive in, a variety of scaffolds have been used. Poly-hydroxyl acids such as PLA and poly lactic-co-glycolic acid (PLGA) have been extensively used in particular because of their properties of bulk-degradation by hydrolysis, which provides a degradation profile to match tissue in-growth (Fig. 2). In this study, PLGA is utilized as the base scaffold for the cell-loaded hydrogel to be infused into. It will serve as the stable environment that the cells migrate and attach to once the hydrogel phase has degraded.

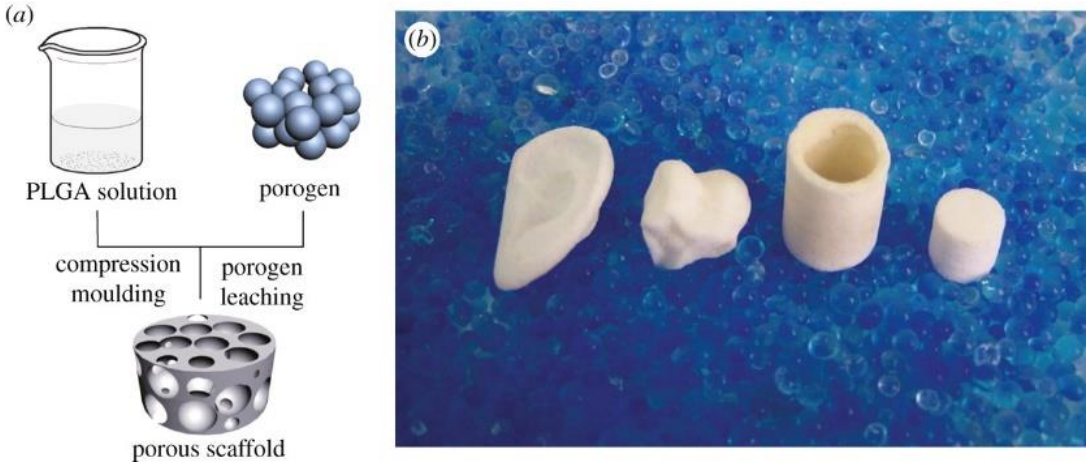

Fig. 2. Standard preparation of and examples of PLGA Scaffolds *(32)*

Finally, a hydrogel is necessary in order to maintain the cells in a local environment of the scaffold, or in other words – to increase cell efficiency. Hydrogels, within the field of tissue engineering, offer a three-dimensional environment for cell growth, water for hydration, and tissue-like mechanical behavior *(7-9)*. Primarily, hydrogels have been classified as either synthetic or natural, based on the biomaterial used to form the hydrogel *(1, 10)*. Poly ethylene glycol, poly vinyl alcohol and poly alpha-esters are some of the commonly used synthetic hydrogels *(11-13)*.

They offer synthetic flexibility and property modulation, which is key in designing hydrogel matrices with specific physical and mechanical properties *(8,1)*.

Natural hydrogels are widely used in tissue engineering due to their excellent biocompatibility *(14-16)*. Developed hydrogels have been a part of numerous drug delivery and tissue engineering strategies. Some known examples of natural hydrogels include collagen, elastin, and fibrin, amongst which fibrin stands out as it can be derived from the patient itself *(17-22)*. Fibrin has been known and used as a sealant for more than fifty years *(23)*. Fibrinogen protein is combined with animal derived thrombin to form fibrin hydrogel, in a manner that mimics the coagulation cascade *(24-26*). In response to tissue injury inside or on the skin of the body, fibrin clot formation is of great focus throughout the process of hemostasis. Overall, the most relevant step of hemostasis for this study is that thrombin converts the soluble fibrinogen into insoluble fibrin strands. These strands are then cross-linked by factor XIII to form a blood clot. And thus, the clot acts as a hydrogel. However, even though the animal-sourced thrombin effectively converts fibrinogen to fibrin, it carries the risk of disease transmission when utilized with real life patients *(27)*. The fibrin-containing tissue engineered device also requires FDA approval before surgical use *(27)*.

Therefore, there is renewed interest in patient-derived biomaterials, which, by virtue, are derived from the patient and can be implanted into the same patient. Patient-derived biomaterials, unlike existing materials, have the added advantage of surgical use without FDA approval *(28)*. To our knowledge, there are only two such materials: blood-derived fibrinogen, and hair-derived keratin *(29, 19)*. In this study, we have developed a completely automated protocol to isolate and form fibrin hydrogel from the patient's peripheral blood. An automated device is used to obtain plasma from the blood in a reproducible manner *(30-31)*. Moreover, this protocol develops a

method to gelate plasma into fibrin without the use of thrombin, but instead using calcium in the form of calcium chloride. The physiological basis for using calcium is that it is an incredibly important player in multiple parts of the intrinsic pathway of the coagulation cascade. Essentially, upon injury, the platelets and injured tissue releases thromboplastin, forming the enzyme prothrombinase in the blood. In the presence of calcium, this prothrombinase the inactive plasma protein of prothrombin into active thrombin, which is what has been utilized to create fibrin hydrogels in the past. Now, by utilizing just calcium, the pathway continues naturally. Upon activation, the thrombin converts soluble fibrinogen into insoluble fibrin which forms a "clot" or for our purposes – a hydrogel. This study presents a completely intraoperative protocol to derive fibrin from peripheral blood next to the bedside for tissue engineering use.

Ultimately, loading patient-derived stem cells into a patient-derived hydrogel reduces risk of infection, rejection, and FDA intervention significantly. The fibrin hydrogel initially serves as an efficient cell loader, "trapping" the cells through the fabricated clotting mechanism. As a sacrificial layer, however, the hydrogel biodegrades within 2-3 days. As cells need stability, they then migrate and attach to the bioengineered scaffold. Thus, the hydrogel works to improve cell loading and retention, and therefore overall efficiency. Upon successful cell differentiation, this engineered biomaterial can be utilized as a tissue engineering strategy to heal bone injuries through regeneration of tissue at the injured area.

Problem Statement

Tissue engineering has developed ways of regenerating bone by infusing osteoblastic stem cells into autografts, allografts, or hydrogel-scaffolds. However, there remain two primary needs for optimizing tissue engineering strategies for healing osteochondral defects:

1. Increase cell loading and retention rate upon delivery of the cells in-vivo.
2. Decrease risk of infection and rejection of transplant for the patient.

A patient-derived hydrogel meets the first need as it can be combined with a scaffold to transplant cells so that they remain at the point where regeneration is necessary. As the hydrogel is engineered using materials from the patient, for the patient, and all within the surgical operation room, the biomaterial also meets the second need, while eliminating the need for FDA approval.

Methods

All procedures were carried out at room temperature, unless otherwise indicated. The overall procedure encompasses three main stages. The first stage is (I) hydrogel preparation, the second is (II) mixing of stem cells into the hydrogel, and finally, the third stage is (III) infusion of the cell-containing hydrogel into the PLGA scaffold, which will determine increased efficiency of cell loading [Fig. 4].

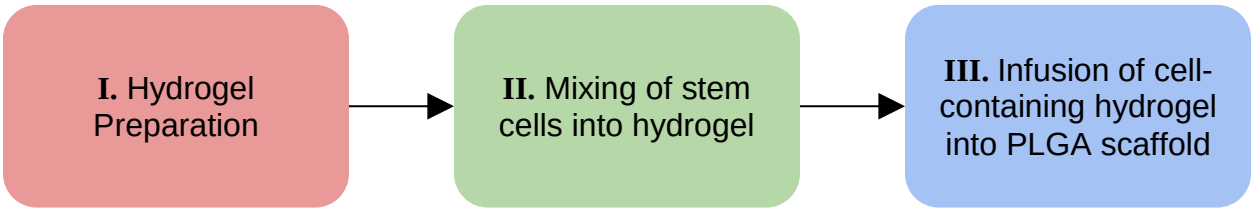

Figure. 4. Overview of experimental methodology

I. Hydrogel Preparation

1. Platelet-Poor Plasma (PPP) Isolation

Autologous human peripheral blood was drawn into an anticoagulated 60 mL syringe. The human peripheral blood was obtained from a commercial source (Zenbio inc.) that was shipped in a tube containing anticoagulant of the same day the blood was drawn, and then received the day after. First, the 60 mL syringes were rinsed with ACD-A solution (anticoagulant). The ACD-A solution was pipetted up and down so as to coat the inside surface of the syringe. The remaining ACD-A solution was recycled. The blood sample was transferred into one of the ACD-A coated syringes and the other syringe was kept empty for later use. The purpose of adding ACD-A to the syringe is to hinder the process of blood clotting. Therefore, using a syringe that has not been coated with ACD-A could result in early gelation and skew the

results.

The Magellan System needs about a minimum of 30 mL to operate, however optimal results were achieved with 40-60 mL of starting volume. The instructions for the use of the Magellan System were included with the disposable kit, and thus followed for this study (Fig. 5).

The centrifuge and syringe covers were slightly opened to load the device. The disposable separation chamber was placed into the caddy of the centrifuge by first maneuvering the tubing through the canal of the base. The separation chamber is then locked in place by gently pushing on both ends of the cylindrical surface, and the tubing is funneled through the tubing clamp and grooved fixture that is leaving the centrifuge housing. Once the centrifuge cover was closed, the green light indicated successful locking. Then, the long and short tubing were attached to the provided 60 and 10 cc BD syringes, respectfully. The small 10 mL BD syringe was connected to the tubing and placed into the syringe position for the device to perform. Then, platelet-rich plasma (PRP) was collected in this syringe. ACD-A was not needed in the 10 mL syringe. The long tubing was funneled through the pinch-valve under the syringe cover. Then the syringes were placed into the syringe handles and the syringe cover was closed. The green light then indicated successful assembly and closure. Any errors in assembly would be indicated by red lights instead of green.

The final desired volume of concentrate (3-10 mL) was selected using the “+” and “-” buttons. The green “play” button was pressured, then “PPP” to begin processing the blood. The Magellan System is a dual spin processing device. After a soft spin to concentrate a packed Red Blood Cell (RBC) layer, the packed RBCs were removed and collected back into the existing 60 mL BD syringe. While the device enters into the hard spin to create the PRP, the RBC filled syringe was removed. While the device enters into the hard spin to create the PRP, the RBC

filled syringe was removed and replaced with the empty 60 mL BD syringe previously coated with ACD-A and then the process was "started" again. The new syringe will collect the Platelet-Poor Plasma (PPP) to be used for the following steps. The first syringe was disposed of appropriately. Then, once the cycle has been completed, the syringe was removed from the machine. PRP will be collected into the 10 mL BD syringe.

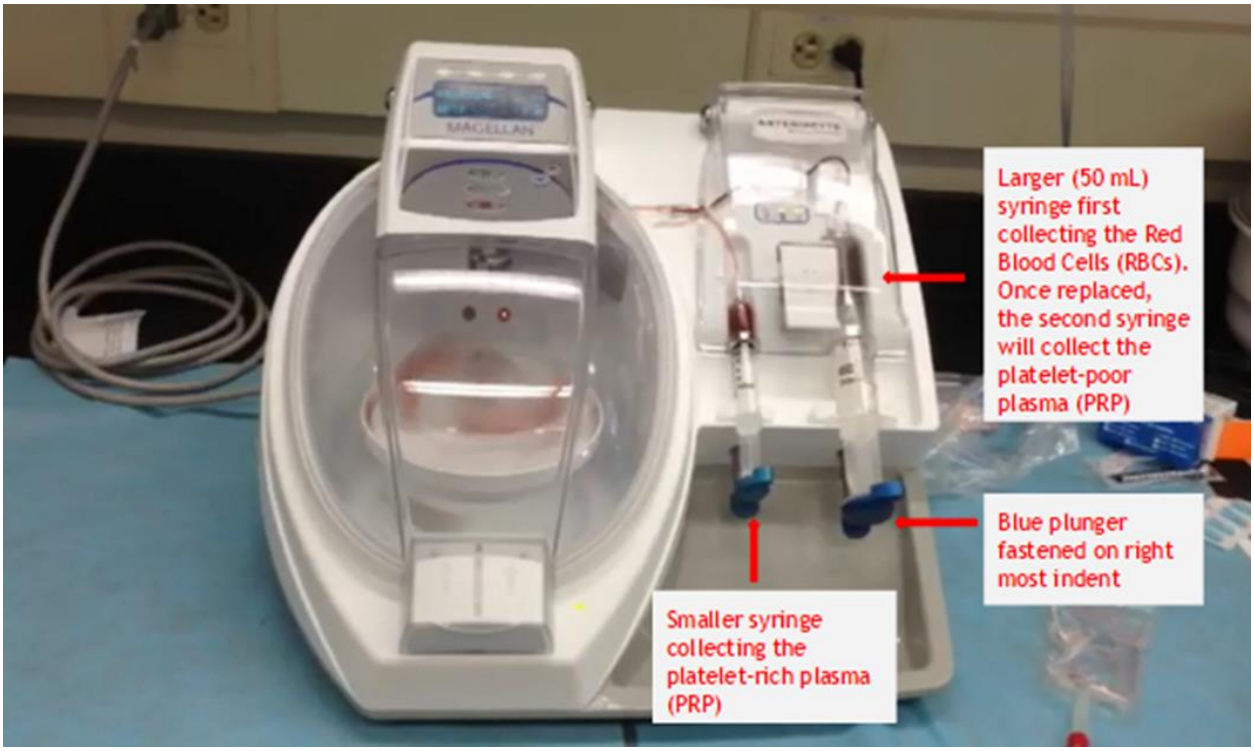

Figure 5. MAGELLAN® blood processor was used to process human peripheral blood – it is a completely automated and close-looped device with the ability to separate blood into three fractions: red blood cells, platelet-rich plasma (PRP), and platelet-poor plasma (PPP). The latter fraction was used to form fibrin gel by mixing with an appropriate amount of calcium chloride solution.

2. Fibrin Gel Preparation

Initial preparation for the fibrin gel was creating the $CaCl_2$ mixture. Five aliquot tubes were obtained to be filled with the same amount of PPP, which will now be referred to as plasma. Various concentrations of 100 mM $CaCl_2$ were filled in order to determine the optimal

concentration of calcium for gel formation. The appropriate amount of 100 mM $CaCl_2$ was weighed out for a 5 mL stock solution. Then, five different concentrations of $CaCl_2$ were determined and each of the aliquot tubes was labeled with the corresponding volume.

The plasma gelation to form fibrin begins by adding 100 uL of the plasma to the first aliquot tube. The plasma was pipetted up and down before transferring it to the aliquot tube in order to ensure a homogenous and even amount of fibrinogen in each tube. After adding the appropriate volume of $CaCl_2$, the timer was started immediately. By rotating the tube towards a 90 degree angle, the point at which a distinct difference between gel and liquid is observed can be determined. This is the time required for gelation. It was important to not miss the window of the initial gel formation. This was the critical time that differs between various calcium concentrations. Other than the point at which the liquid is seen to diffuse through the forming gel, another important observation is when the tube is completely flipped over (180 degrees) and the gel stick to the top. At this point, a significant amount of gelation has occurred such that the substance “sticks” to the top. The procedure ought to be repeated for the five remaining aliquot tubes and the time required for gelation recorded. The overall procedure of obtaining fibrin gel is depicted through a block diagram in Fig. 6**.**

Data Analysis: The time required for gelation should be analyzed for each of the aliquot tubes and the optimal concentration of $CaCl_2$ determined. Depending on who is performing the experiment, the optimal value may be different. In a real world application, the optimal concentration of $CaCl_2$ is that which gives the surgeon enough time to transfer the mixture of plasma and calcium into the body before gelation has initiated.

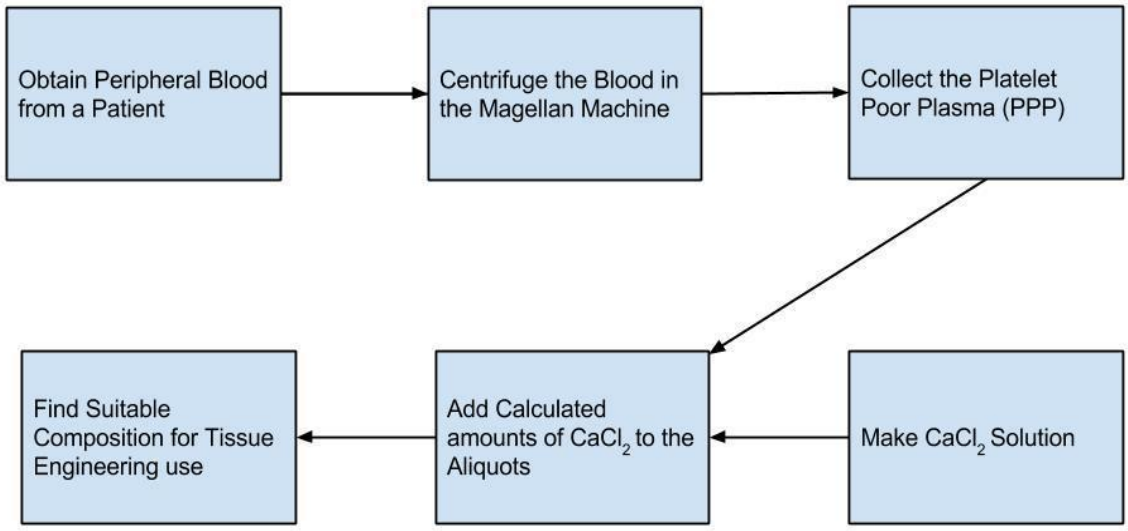

Figure 6: Scheme showing the steps utilized to prepare fibrin gel from human blood-derived plasma. Plasma was obtained by processing peripheral blood via Magellan system followed by calcium-mediated gelation.

II. Mixing of stem cells into hydrogel

3. Cell Culture and Counting

MC3T3 mouse pre-osteoblastic stem cells were the type of cells cultured and utilized for the study. The media for the cells was changed every 48 hours (Monday, Wednesday, and Friday).

The materials needed for each plate of cell culture include the following:

- Media
    - Minimal essential medium (500ml)
    - Fetal bovine serum (FBS) (50ml) -10%
        - FBS has low antibodies and lots of growth factors
    - 100 U/mL penicillin (5ml)-1%

The cell plates were checked for confluency before passing and counting. If the plate was confluent, then the cell passage protocol was followed. The media was first aspirated, and the

plate was wash with 10ml PBS to remove any excess media. 7-10 mL trypsin was added to the plate in order to break the bonds between the cells and the plates so they could be transferred later on. After adding trypsin, the plate was incubated at 37ºC for 7 minutes. An equal amount of media was added to stop the effects of trypsin. Then, the cells with media were removed from the plate and pipetted into a tube for centrifugation. Another tube with equal amount of water was filled and they were centrifuged at 1300 RCF for 7 minutes.

After centrifugation, the excess media on top was aspirated, and then the packaged cells at the bottom of the tube were pipetted out without being disturbed. The cell counting protocol was followed using a hemocytometer and microscope. First, the cells were resuspended in 1mL medium, then 60 µL of that solution was transferred into an Eppendorf tube. Finally, 10 µL of that cell suspension was mixed with 90 µL of trypan blue (1:10 dilution). This was injected onto the hemocytometer for cell counting (Fig. 7A).

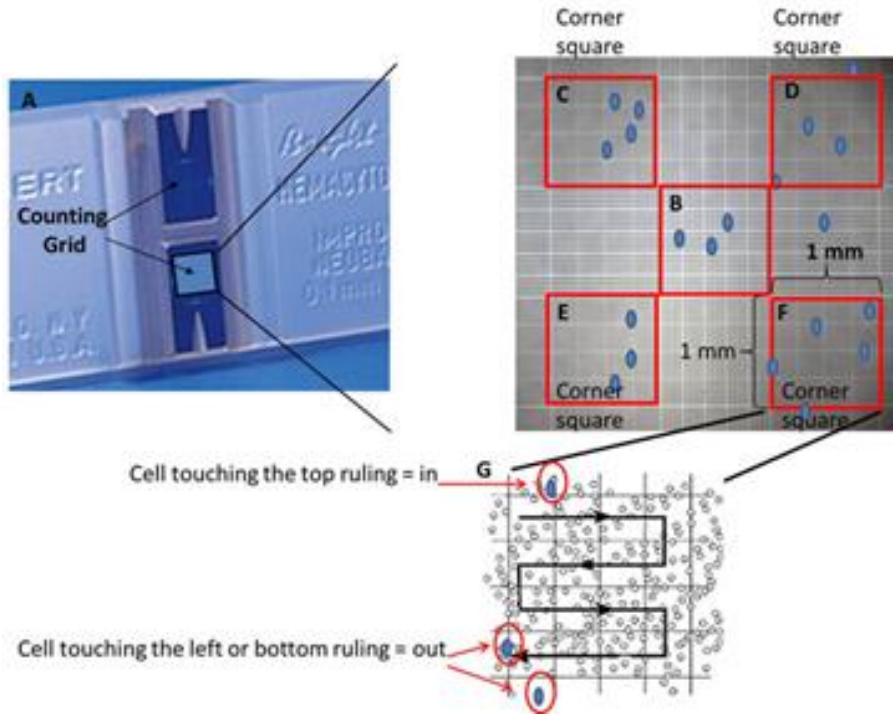

Fig. 7. Cell counting protocol

As depicted in Fig. 7C-F., the cells in each of the four corners were counted. Cell concentration, **C**, (per mL) was determined by Eq. 1.

$$\mathbf{C} = \text{total cell count in 4 squares} * 2500 * \text{dilution factor (10)} \quad \textbf{Equation 1}$$

0.6 Million cells were then replated for further culture and passage.

4. Assessment of Cell Survival and Growth

200K cells were injected into the hydrogel for characterization of its ability to trap cells. Confocal studies on days 1,3,5,7, and 14 were completed to assess for cell survival and growth within the hydrogel. The first sample was observed 24 hours after cell seeding, and each sample after was observed at the appropriate time points.

III. Infusion of cell-containing hydrogel into PLGA scaffold

5***. MTS Standard Curve***

The cell passing and counting protocol was followed, after which the exact total amount of cells numbers for the MTS assay (duplicate) and scaffold experiment was calculated. For the MTS assay, 6 wells with different cell numbers were set up (Table 1). The corresponding volume of cell numbers were added into individual wells along with 1mL of media. After adding 200uL of celltiter 96 to each well, pipetting it up and down, the plate was wrapped with foil and incubated at 37 degrees Celsius for 2 hours. The cell metabolic activity was assessed by a colorimetric assay. The cell number in each well was therefore determined by its relative metabolic activity. At the end of the incubation, the reaction was stopped by adding 250 uL of 10% SDS solution to each well. After 5 minutes, 1 mL of each well was moved into a new plate

to keep the volume equal. The absorption level was then read and recorded to create the standard curve.

Cell #	50K	100K	150K	200K	250K	0K

Table 1. Different cell numbers were set up for the MTS assay in order to establish a standard curve

6. ***Determination of Cell-Loading Number***

An important aspect of this study was to determine how cell retention changes with an increasing initial number of cells loaded. The hybrid hydrogel-scaffold biomaterial was loaded with 50K, 200K, and 500K cells, and the resulting cell retention was determined using MTS assay. This was compared with 200K on the scaffold alone, as well as 200K on the fibrin gel alone.

7***. Determination of Optimal Cell-Retention***

The following protocol was followed in order to determine whether the hybrid hydrogel-scaffold environment retains more cells than the scaffold alone. PLGA scaffolds were first developed and sterilized. Various forms of the biomaterial were then arranged within a 24 well plate (Table 2). MTS Assay was used to determine concentration of cells in each well. The readings function on basic spectrophotometry, with the wavelength set to 490 nm.

				Control
Set 1	Scaffold+ hydrogel +200K cells	Scaffold+ hydrogel +200K cells	Scaffold+ hydrogel +200K cells	scaffold+ hydrogel (no cells)
Set 2	Scaffold+ 200K cells	Scaffold+ 200K cells	Scaffold+ 200K cells	Scaffold (no cells)
Set 3	Hydrogel + 200K cells	Hydrogel + 200K cells	Hydrogel + 200K cells	Hydrogel (no cells)

Table 2. Cell loading efficiency was determined by comparing the different scaffold and hydrogel setup results from the wells.

For Set 1, 34uL of the plasma was mixed with 200K cells, and then 6uL $CaCl_2$ (100mM) was added to the tubes. After mixing the solution, it was immediately loaded onto the scaffolds in the 24 well plate. 1 mL of medium was added and then incubated for 1 hour, after which the well was read using MTS assay.

For Set 2, 40 uL of medium was mixed with 200K cells and then loaded onto the scaffolds in the 24 well plate. 1 mL of medium was added and incubated for 1 hour. After incubation, the medium was removed, and 1 mL of PBS was added to the side of each well and to rinse the scaffold, and then finally aspirated. The scaffold was transferred into a new 24 well plate, 1 mL of medium and 200 uL of celltiter 96 was added, then incubated for 2 hours. The reaction was then stopped by adding 250uL of 10% SDS solution. Each of the well solutions were diluted 1:2, making sure only 1 mL is ultimately being read by the MTS assay.

For Set 3, 34uL plasma was mixed with 200K cells and then 6uL of $CaCl_2$ was added to the tub and mixed. This is to keep the concentration of $CaCl_2$ at the optimal 15mM (Table 3). The cell, calcium chloride, and plasma gel were immediately loaded onto the scaffolds in the 24 well plate. 1mL of medium was added and then incubated for 1 hour. After taking out the medium, 1 mL of PBS was added to each side of the well and pipetted up and down to remove any cells that are not attached. Then 1mL of medium was added to each well, along with the 200uL of celltiter 96 for the MTS assay. The reaction was stopped as for the previous set and then diluted 1:2 before reading.

Results

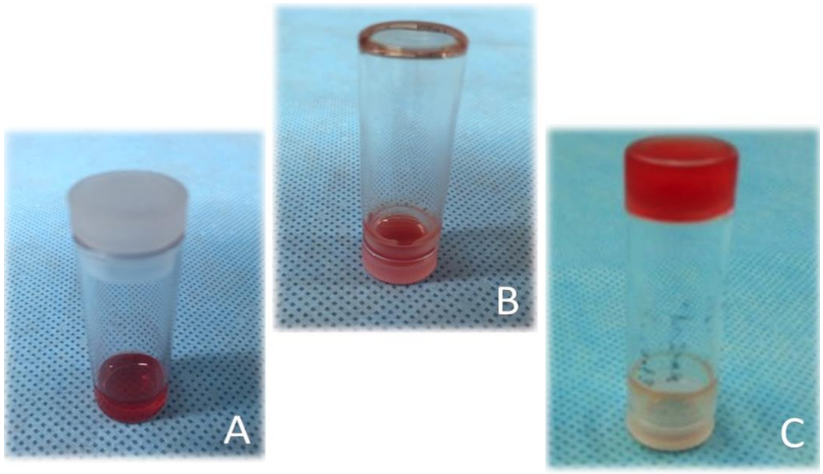

Figure 8: Human peripheral blood derived plasma before and after gelation: (A) Plasma in tube. (B) Plasma inverted before gelation. (C) Plasma inverted after gelation process completed. The hydrogel is viscous and adheres to the top surface.

Calcium Molarity	Gelation Time
.15	~120 seconds
.25	~90 seconds
.35	~80 seconds
.45	~50 seconds
.55	~40 seconds
.65	~30 seconds
.75	~10 seconds

Table 3: Determining Optimal Calcium Molarity Based on Gelation Time

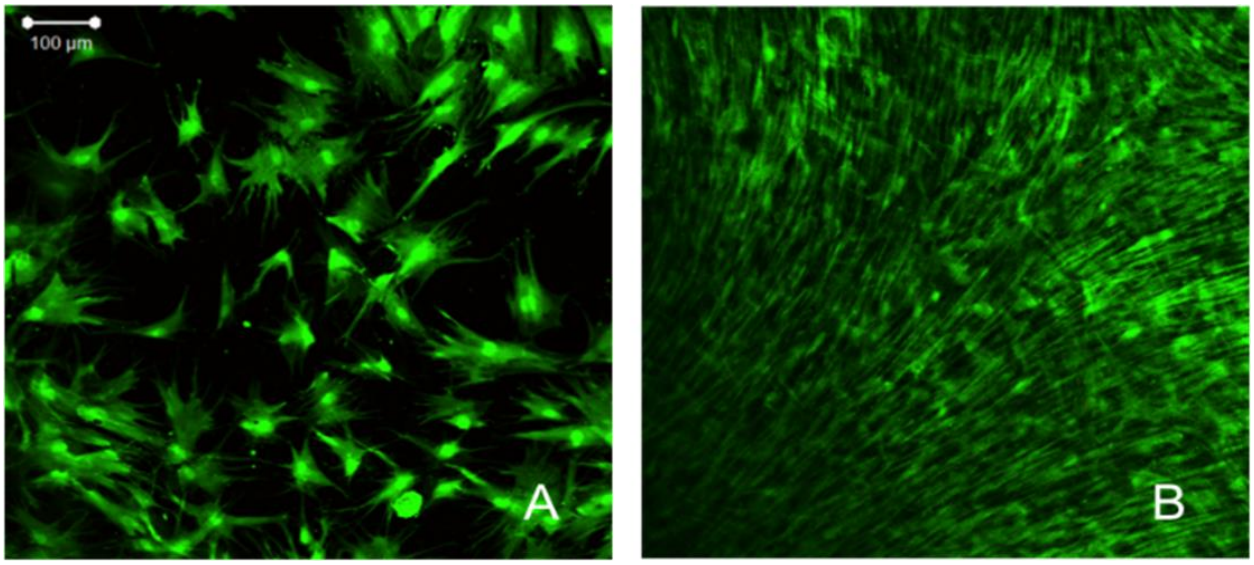

Figure 9. Confocal microscope images of human bone marrow stromal cells (hBMSCs) in fibrin hydrogel cultured for: (A) 1 day, and (B) 14 days.

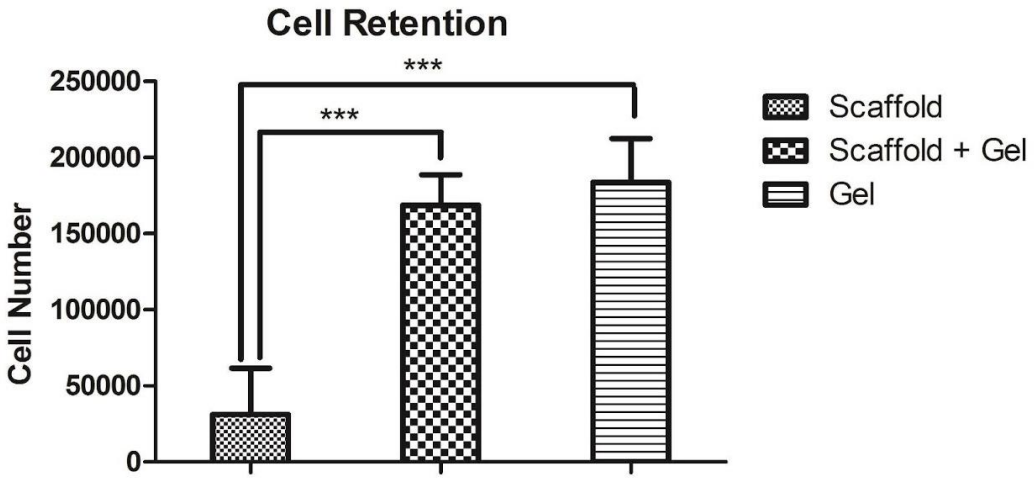

Figure 10.
Comparison of cell retention rate for three combinations of biomaterials from MTS assay data

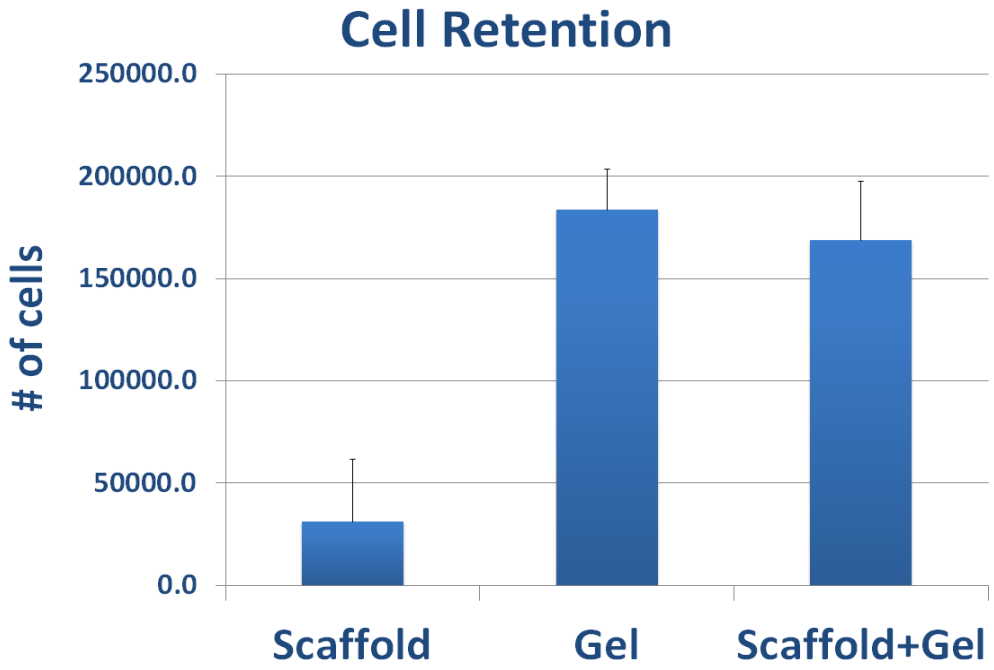

Figure 11: When loading 200k MC3T3 cells, it is observed that the scaffold with the fibrin gel supports cell retention by 91%, as compared to 16.93% by the scaffold alone.

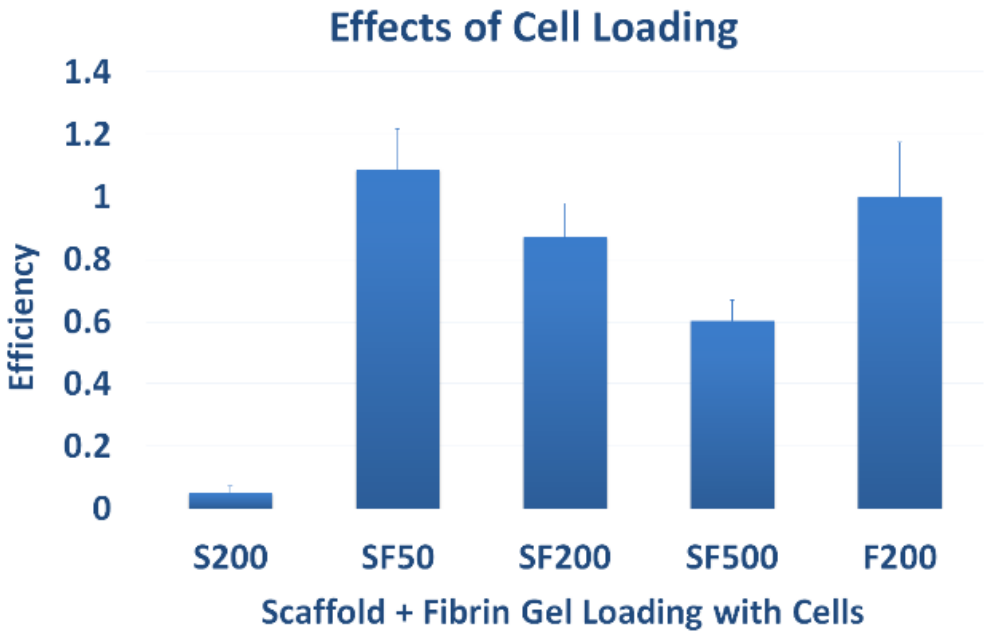

Figure 12: When loading the fibrin-scaffold with 50k, 200k, and 500k MC3T3 cells, it appears that an increasing cell number causes decreased cell retention.

Discussion

The primary goal of this study was to engineer an autologous fibrin hydrogel in a manner that gives the surgeon enough time to process it intraoperatively (Fig. 8C). Thus, it was necessary to complete the $CaCl_2$ optimization study in order to determine what concentration of $CaCl_2$ lends enough time to process the gel before gelation is initiated. A calcium chloride concentration of 0.15 M/L was utilized to form the gel, as it gives approximately 120 seconds to transfer the mixture of plasma and calcium into the body before gelation has initiated (Table 3). In a real world application, the surgeon needs to have enough time to both process and place the hydrogel within the patient's body before gelation. Depending on who is performing the experiment, the optimal value of $CaCl_2$ may be different. Ultimately, the $CaCl_2$ optimization study thus demonstrated that the surgeon can optimize the $CaCl_2$ concentration to reflect the time needed to develop the hydrogel in the operating room. In doing so, the gel can be successfully processed and placed within the patient before gelation has initiated.

The next step was to characterize the autologously formed fibrin hydrogel for cell survival and proliferation. The confocal image recorded on day 1 (Fig 9A) established well spread morphology, which is indicative of the healthy nature of the hBMSCs within the hydrogel. By day 14, the cells became confluent, which confirmed the survival and growth of cells in a calcium-mediated fibrin hydrogel. The cells at this point are those on the petri dish only, as the hydrogel has completely degraded. These images suggest that the completely autologous fibrin hydrogel supports not only cell survival, but also growth.

After engineering and assessing the supportive nature of the fibrin hydrogel, it is thus confirmed that stem cells can survive and grow in the environment that it provides. The main goal of the fibrin hydrogel as a sacrificial matrix, however, is to increase cell efficiency.

The fibrin hydrogel is a sacrificial matrix meaning that it degrades overtime, within about 2-3 days. After degradation of the hydrogel, the cells seek a stable environment in order to stay alive, such as a polymer scaffold. Another goal of this study was thus to determine the cell retention rate for the hybrid hydrogel-polymer scaffold.

To do so, the next step is to use the autologous hydrogel to efficiently load cells onto a three-dimensional and porous matrix that eventually serves as a long-term stable environment for the cells. The gel was infused into a 3D PLGA matrix to form a polymer-gel hybrid matrix *(33)*. We used MTS assay to quantify the cell loading efficiency based on the cell concentration in each of the 24 wells. The results show that the concentration of the cell-containing gel wells was the highest (Fig. 10). Thus, the cell-containing gel is the most effective in retaining cells. This is expected as the fibrin gel is engineered in order to "trap" the cells more effectively. The cell-containing gel within the scaffold decreased in cell retention as the scaffold was not as static an environment for the cells. It was also more difficult to remove all the cells from the scaffold in order to optimally read the concentration from the MTS assay, which may have caused slight alterations in the data set. As expected, the scaffold alone resulted in the smallest number of cells retained because it lacked the hydrogel that was responsible for initially "trapping" the cells and giving them a stable environment to survive in. The hydrogel was also an important mechanism of delivering the cells to the scaffold, without which cell retention would decrease.

Ultimately, the cell retention rate increased for the hybrid hydrogel-scaffold as compared to the scaffold alone. In fact, when loading 200K MC3T3 cells, it was observed that the scaffold with the fibrin gel supports cell retention by 91%, as compared to 16.93% by the scaffold alone (Fig. 11). Thus, it is determined that the hydrogel improves cell efficiency by delivering and supporting the cells until degradation, after which the scaffold serves as a longer-term support.

Moreover, upon using MTS assay, it was also determined that an increasing cell number actually causes decreased cell retention within the hybrid hydrogel-scaffold biomaterial (Fig 12). This may be a result of decreased space for each individual cell when there is an increased number of cells in the same environment. Real clinical conditions may call for increased cell loading numbers to be most effective in treating patients. However, for the purpose of laboratory studies with smaller sized scaffolds, it is optimal to not use too large of a cell loading number in order to increase cell retention within the biomaterial. Thus, 200K cells served to be the optimal number, as it was not too small that it would deviate from realistic clinical applications, but also not too large to significantly decrease cell retention rates.

Conclusion

Using an automated and closed-loop machine, we were able to first develop a reproducible method to engineer an autologous hydrogel from human peripheral blood. By mimicking the coagulation cascade, this calcium-based fibrin gel "clots" into a biodegradable hydrogel. Utilizing such a patient-derived biomaterial will immensely decrease the risk of infection as compared to other thrombin-based hydrogels. It will also decrease risk of rejection as the hydrogel is formed from the patient's own peripheral blood, and implanted with the patient's own stem cells. Most importantly, this hydrogel can be engineered in a manner such that the surgeon has enough time to produce and implant it within the patient in the operating room.

The cell retention rate for the hybrid hydrogel-PLGA scaffold loaded with 200K cells increased from about 16.93% cells for the scaffold alone to 91% cells with the hydrogel. Thus, this study confirms that by forming a hybrid hydrogel-polymer matrix, the sacrificial gel phase will increase cell retention. As the hydrogel degrades upon 2-3 days of implantation, the cells must migrate to the more stable environment of the biocompatible PLGA scaffold. Long-term 21-Day confocal studies are needed to confirm that after the cells migrate to the scaffold, they remain attached, and that there is continued growth and confluency.

Ultimately, then, utilizing this patient-derived hydrogel will first improve cell-loading, retention, and thus overall efficiency without the risk of infection or rejection of the cells in-vivo. Then, after degradation, the hydrogel as a sacrificial matrix will also allow for cell migration onto the stable scaffold. Therefore, the use of this patient-derived hydrogel has numerous applications in tissue engineering strategies, particularly in improving the cell loading and

retention rates onto a scaffold next to the bedside. This study has established that the patient-derived biomaterial could be used to efficiently seed 3D matrices at the bedside.

Future improvements include modifying the procedure to further work towards a vision of completely intraoperative tissue engineering strategies (CITES) (Fig. 13). In particular, while confocal studies have proven that the hydrogel can support cell viability and growth, the 21-Day confocal study of the hybrid hydrogel-scaffold will provide a greater understanding of long-term cell survival and osteogenic differentiation within the complete biomaterial. Testing on various sizes and shapes of the scaffold can also lend insight into possible individualized biomaterials based on the exact bone defect(s) the patient has. Improvements leading to this vision can then develop a modified method of engineering the biomaterial that can be easily translated into the clinic for surgical use.

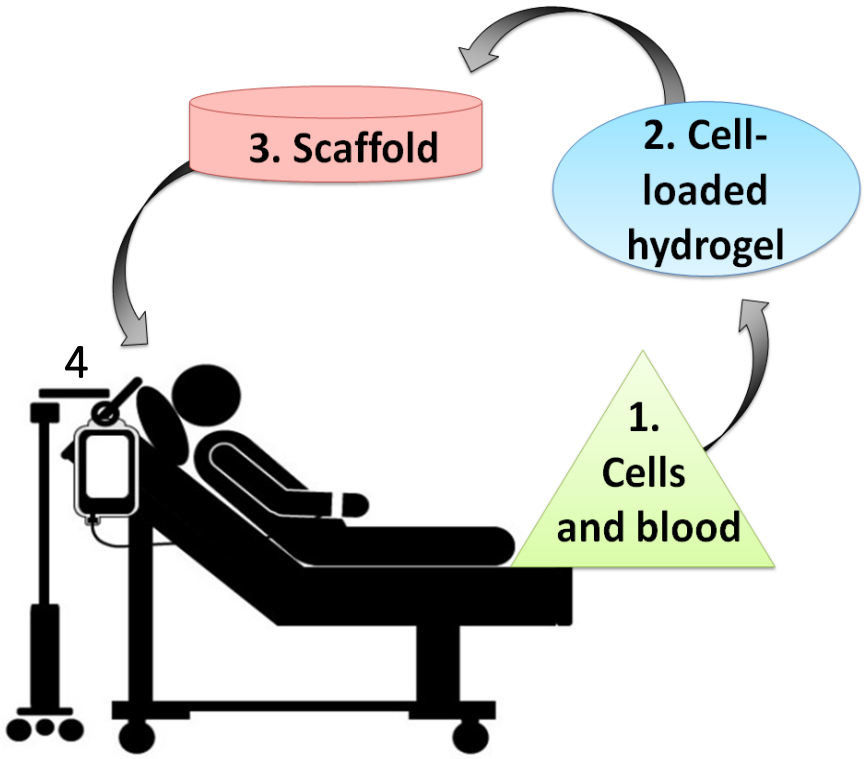

Fig. 13. Vision:
To develop a Completely Intra-operative Tissue Engineering Strategy (CITES) that can be easily translated into the clinic for surgical use.

1. Obtain patient's bone marrow aspirate (BMA) and peripheral blood (PB)
2. Derive stem cells from BMA and biomaterial from PB
3. Develop cell-loaded biomaterial as an injectable scaffold
4. Implant intra-operatively

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