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DEVELOPMENT AND OPTIMIZATION OF A BIOCOMPATIBLE HYDROGEL FOR SKIN REGENERATION COMPOSITES.

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1. ABSTRACT:

Tissue engineered skin substitutes have evolved tremendously in the past couple of years and newer procedures are being developed every day to generate skin substitutes for clinical applications. These skin substitutes have been finding widespread application, especially in the case of burns and skin-related disorders where the major limiting factor is the availability of autologous skin. With the improvement of tissue engineering technology, the use of artificial skin to repair various skin defects and wounds is slowly moving into the clinic. The key to this technology however, lies in the development of dermal substitutes. Hence, the main focus in developing a skin substitute is to mimic the dermal layer of the skin. Although, to this date, a complete functional skin substitute in which the dermis can vascularize rapidly is not available. One of the important components in mimicking the dermis is the hydrogel that is used for the skin regeneration composite. The main properties of the hydrogel that make it essential for incorporation into the scaffold include biocompatibility, good stability and modulus, ability to form a homogenous gel and cost-effectiveness. Therefore, this research focuses on the development and optimisation of a biocompatible hydrogel for the skin regeneration composites.

2. INTRODUCTION:

The skin is the largest organ of the human body, representing approximately one-tenth of the body mass, and is necessary for survival. This organ serves several important functions, including physical barrier to the external environment, thermal regulation, and retention of hydration. Due to the presence of stem cells in the skin, the epidermis is able to stimulate self-regeneration on acquiring a wound. However, in case of deep injuries and burns, this process of healing by the epidermis is not enough, thus leading to a chronic wound. Loss of skin more than 4cm in thickness requires grafting for its treatment (Herndon et al., 1989). Surgical treatments available for skin healing have very limited availability of donors. Additionally, the use of foreign tissue poses a high risk of rejection. For the defect of skin and soft tissue, the traditional treatment methods such as medium or full-thickness skin flap transplantation are effective in wound repair, however, still pose complications such as graft failures, infections, poor healing, tissue contraction and scarring (Li et al., 2021).

As the use of artificial skin to repair various skin wounds is gradually becoming clinical, the key technology of skin tissue engineering lies in the development of dermal substitutes rather than the regeneration of skin appendages (such as hair, nail and glands) (Wong & Chang, 2009). Various strategies have been attempted to re-establish the native skin without compromising its functions With newer procedures being developed to generate dermal substitutes, the high cost for developing these substitutes becomes a major concern. Selecting the appropriate materials to work with to mimic the dermal layer can significantly lower the existing costs. One of these materials are the hydrogel which will be integrated into the mesh-like structure that is printed using melt electro writing. Hydrogels are a unique group of biocompatible 3D polymeric substances which can act as a scaffold and mimic the properties of various tissues in the body (Mantha, S., Pillai, S., Khayambashi, P., Upadhyay, A., Zhang, Y., Tao, O., Pham, H. M., & Tran, S. D. (2019). Smart Hydrogels in Tissue Engineering and Regenerative Medicine. Materials (Basel, Switzerland), 12(20), 3323. https://doi.org/10.3390/ma12203323.). The mechanism is to incorporate cells in their structure while the gel degrades eventually leaving healthy tissue behind. Developing and optimising the hydrogel requires it to undergo mechanical and cell compatibility tests so that

the hydrogel can be deemed appropriate for its respective applications and experiments. One such hydrogel that was synthesized and modified using various techniques for this research is GelMA. The base structure of GelMA is composed of gelatin, which provides inherent biocompatibility, including appropriate cell adhesion sites (Liu & Chan-Park, 2010). The adjustment of the various parameters during GelMA synthesis allows regulation of the final mechanical and chemical properties of the resulting hydrogel. Gelatin methacrylate (GelMA)-based hydrogels have proven to show great bio functionality and mechanical tenability which makes it a good option for use in developing skin substitutes (Xiao et al., 2019).

2.1. ANATOMY AND FUNCTIONS OF SKIN:

The skin is the largest organ of the body and accounts for about 15% of the total body weight. Functioning as the exterior interface of the human body with the environment, skin acts as a physical barrier to prevent the invasion of foreign pathogens and provides protection against physical and chemical agents. The skin is made up of three main layers, namely, the epidermis, the dermis and the hypodermis with the epidermis being the outermost layer (figure 1). The stratum corneum is the outermost layer of the epidermis and extends from the so called basement layer to the dermis. The epidermis consists of keratinocytes, melanocytes and the Langerhans cells all specifying in a particular function respectively.

The dermis is the thickest of the three layers of skin and is present between the epidermis and the subcutaneous layer. The dermis is fibrous and elastic in nature which makes this layer responsible for providing strength and flexibility to the skin. It is mainly composed of collagen and elastic tissue among other extracellular components that include hair follicles, glands, nerve endings and vasculature. The structure of the dermis provides a connective tissue framework that aids in protection of the underlying organs and structures (Vig et al., 2017). Due to the presence of nerve ending the dermis also aids in sensation via several mechanoreceptors. Fibroblasts are the primary cells that make up the dermis although histiocytes, mast cells, and adipocytes play important roles in maintaining the structure and function of the dermis as well. The fibroblast cells of the dermis play a major role in the synthesis and production of collagen, elastic and reticular fibres and extracellular matrix material. Dermal adipocytes assist in hair follicle regeneration and wound healing (Kruglikov IL, Scherer PE. Dermal Adipocytes: From Irrelevance to Metabolic Targets? Trends Endocrinol Metab. 2016 Jan;27(1):1-10. doi: 10.1016/j.tem.2015.11.002. Epub 2015 Nov 29. PMID: 26643658; PMCID: PMC4698208.; Vig et al., 2017).

Below the dermis lies the hypodermis layer. Its main function is to provide insulation and cushioning between the skin and skeletal structures such as bones and muscles.

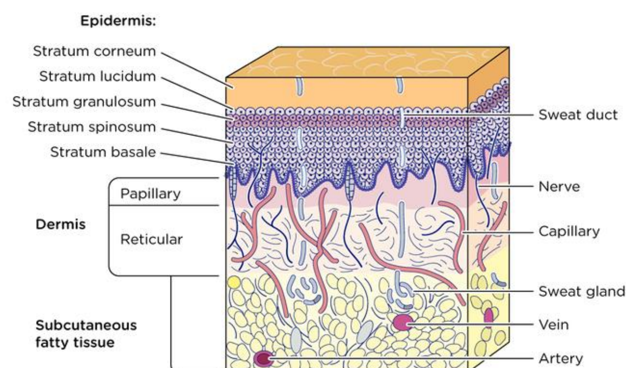

Figure 1: Skin anatomy

2.2. SKIN INJURIES AND DEFCTS:

Skin injuries are damage caused to the skin either on the surface or those that go through the skin to the fat tissue. Most superficial skin injuries are treated with antibiotics and wound dressings. Skin injuries and wounds that require reconstruction and replacement of skin tissue can be classified into the following types: deep burns, lesions from a skin disease, large open wounds, skin cancer surgery, pressure injuries or bed sores and ulcers on the skin that fail to heal. Large open wounds and deep burns take about three months or more to heal after treatment although 34% of these wounds are accompanied by infection (Kruglikov IL, Scherer PE. Dermal Adipocytes: From Irrelevance to Metabolic Targets? Trends Endocrinol Metab. 2016 Jan;27(1):1-10. doi: 10.1016/j.tem.2015.11.002. Epub 2015 Nov 29. PMID: 26643658; PMCID: PMC4698208.; Vig et al., 2017; Yao et al., 2020) (Yao et al., 2020). Third-degree burns destroy the epidermis and the dermis and in some cases the underlying muscles and tendons. The burn site appears red, blistered and swollen. Skin ulcers due to diseases like diabetes and vascular defects last for an average 12 to 13 months but recur in up to 60% to 70% of patients and can lead to loss of function and decreased quality of life, in some cases leading to morbidity (FrykbergRobert, 2015). Damage caused to sweat glands and blood vessels on the skin lead to inability of the skin to carry out its functions. The blood vessels are surrounded by scar tissue and lose the ability to contract properly. Sweat glands cannot produce moisture on the skin surface as they did prior to the injury (Rowan et al., 2015). Due to this, there comes a serious requirement to replace the damaged skin with skin substitutes that can re-establish healthy skin and its integral functions either temporarily in cases where the tissue is not completely destroyed or permanently in cases where the tissue is far from being brought back to its normal functioning.

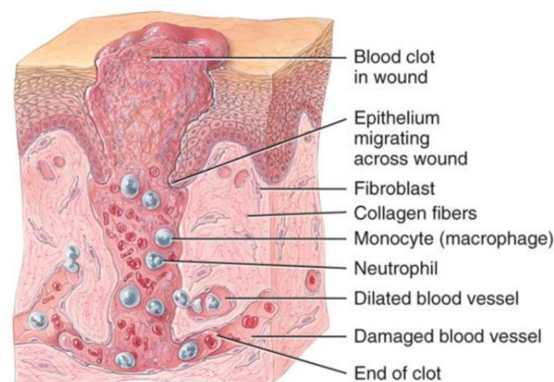

Figure 2: Layers of skin affected in a deep wound.

2.3. CURRENT SKIN SUBSTITUTES:

When a large percentage of skin is lost, epithelial cells cultured into sheets are used to make autologous grafts, which can be lifesaving for patients with extensive burns. On the other hand autologous keratinocytes can be isolated and be transplanted onto large open wounds on being cultured into epithelial sheets (Kruglikov IL, Scherer PE. Dermal Adipocytes: From Irrelevance to Metabolic Targets? Trends Endocrinol Metab. 2016 Jan;27(1):1-10. doi: 10.1016/j.tem.2015.11.002. Epub 2015 Nov 29. PMID: 26643658; PMCID: PMC4698208.; Vig et al., 2017). Epidermal stem cells grown on fibrin matrices or allogeneic dermis have been useful in developing skin substitutes. Epidermal stem cells on

fibrin matrices show the ability to regenerate the normal dermal-epidermal junction and the superficial portion of dermis. However, these epidermal stem cell grafts are not capable of restoring fully functional skin (Wong & Chang, 2009).

Dermal skin substitutes are a heterogeneous group of wound healing materials that aid in closure of the wound and replacement of some functions of the skin (figure 3). They provide a number of biological and physiological properties of human dermis that promote new tissue growth and conditions for healing. The materials used in developing these substitutes re biodegradable and are composed of a bilayer of collagen and glycosaminoglycan (*3D bioprinting for skin tissue*). Dermal matrices are independent of imbibition or inosculation for immediate viability (Dermal Skin Substitutes for Upper Limb Reconstruction: Current Status, Indications and Contraindications.; Rowan et al., 2015). Still, it was found that the newly formed collagen and elastin consists of an abnormal morphology that does not resemble normal skin collagen. This resulted in good clinical outcomes however, complications were reported in the long term. Another area of concern is the high cost associated with dermal skin substitutes that makes it almost unaffordable for a vast majority of patients, especially in developing countries. This high cost results from the materials used to develop the scaffold, clinical trial costs, manufacturing costs and high initial costs that make its access very limited to both patients and healthcare professionals. Thus, it is important to develop a skin model that is inexpensive and accessible by a vast majority of the population due to the large number of skin injures and defects that are reported on a daily basis. Additionally, it is equally important to develop a skin substitute that is of great quality, biocompatibility, has the required shelf life and reduces scarring and swelling.

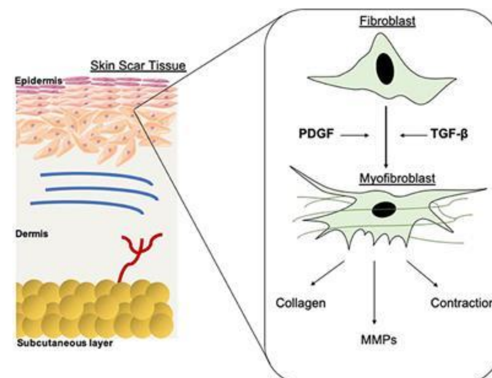

Figure 3: Fibroblast cells in the skin.

3. MATERIALS AND METHODS:

The key technology in the field of skin tissue engineering lies in the development of dermal substitutes. The regeneration of dermis not only solves the issue of shortage of autologous skin sources but also makes the surgery less complex (Rowan et al., 2015; Subramanian et al., 2011). The dermis is the main layer of human skin, having a thickness of 1 mm. The Young's modulus of this layer is about 88 to 300 kPa which is much lower than that of the epidermis (Zhu et al., 2019). A bio scaffold with appropriate physical features, cellular and mechanical properties, such as its elastic modulus and strength is required to develop a dermal skin substitute. All these factors must be in the range of the healthy natural dermal layer to ensure mimicking the dermal layer to the fullest. Additionally, the scaffold is responsible for providing the layer with structural and mechanical properties. This is achieved when a hydrogel and scaffold are mixed together resulting in stiffness of the

scaffold composites to increase synergistically (up to 54-fold), compared with hydrogels or microfiber scaffolds alone (Ramasamy et al., 2021). This increase in matrix stiffness triggers TGF-β signalling in dermal fibroblasts, causing them to secrete more collagen I, which is the main component of skin ECM. The hydrogel must be biocompatible, chemically tuneable, stable with good modulus and suitable for cell culture. A dermal substitute contains allogeneic human fibroblasts cultured in a scaffold. These cells are cultured in the scaffold and are tested periodically for cell proliferation and viability assays.

3.1.STEPS INVOLVED IN ENGINEERING THE DERMAL LAYER:

1. DESIGN AND PRINTING OF PCL SCAFFOLDS:

PCL is a member of the biodegradable polyesters. It is an aliphatic semi-crystalline polymer which has a melting temperature ranging between 59 and 64 °C. This temperature being above the human body temperature makes it an effective material to develop scaffolds with. The scaffolds are printed using melt electro writing technique. Melt electro writing (MEW) is a high-resolution additive manufacturing technique based on the electrohydrodynamic processing of polymers. MEW is used to fabricate scaffolds for biomedical applications where the microscale fibre positioning has substantial implications in its macroscopic mechanical properties (Rizwan et al., 2020). It has the capability to produce fibres in the micro- and nanometer range. The device consists of a print head that has a heating system to melt the material in a syringe. The MEW head uses a computer aided translating system to write on a collector. A delivery system forces the extrusion of the polymer melt through the nozzle. An applied potential difference between the nozzle and the collector leads to an electrical field. This in turn concentrates charges in the fluid drop at the closest point to the collector. The initiation of a jet within MEW depends on the mass flow rate to the nozzle, applied voltage, and collector distance, and can take in the order of several or even tens of minutes, depending on the flow rate and/or melt viscosity. PCL is readily processable via MEW due to its low melting temperature and rapid (Ramasamy et al., 2021).

2. CELL SEEDING:

Here, the fibroblasts proliferate and multiply and during this process secrete matrix proteins, growth factors, human dermal collagen, and cytokines (Bolle et al., 2020). Thus, this creates a 3D human dermal substitute containing active living cells that can be used to cover skin injuries and wounds. The fibroblasts used for engineering the dermal substitute are harvested from neonatal human skin and are cultured in vitro on a polycaprolactone scaffold.

3. SYNTHESIS AND CROSSLINKING OF GelMA:

Hydrogels are hydrophilic polymers with an intrinsic three-dimensional structure. Hydrogel biomaterials are very similar to the extracellular matrix because they exhibit similar mechanical properties to natural tissues. The water content of hydrogels also matches those that of natural tissue up to a good extent. Naturally derived hydrogels are widely used in biological applications because of their biodegradability, cell signalling abilities and cellular interactions. However, these materials are not always ideal for tissue engineering applications because they are usually limited by factors like low mechanical strength, structural degradation or potential immunogenicity (Xiao et al., 2019). This can however be modified

for the better by chemically modifying the hydrogel using various techniques. One such naturally derived hydrogel is Gelatin Methacryloyl (GelMA).

3.1. CHARACTERISTICS OF GelMA:

GelMA is a gelatin derivative, which is in turn a hydrolysate of collagen. Gelatin has a variety of bioactive motifs including arginine-glycine-aspartic acid (RGD). This promotes the adhesion and growth of different cell types and matrix metalloproteinases, which is used for cell remodelling and further enhances the physicochemical properties of gelatin hydrogels (Xiao et al., 2019). However, gelatin shows low immunogenicity due to a low number of aromatic groups. GelMA hydrogels are derived by reacting methacrylic anhydride (MA) with gelatin and by crosslinking in the presence of a photo initiator. The RGD in particular does not contain a functional group that can react with MA . Due to this the cell adhesion properties of gelatin in GelMA materials are retained.

3.2. ADVANTAGES OF GelMA:

One of the major requirements in developing a hydrogel for a skin substitute is that the gel must be cell friendly (figure 4) and suitable for 3D cell culture. GelMA has proven to be biocompatible and very stable with a good modulus which makes it highly advantageous for its use in skin substitutes (Xiao et al., 2019). On using the right synthesis process for this gel, it also yields a homogenous and uniform gel. GelMA, being cost effective compared to most other hydrogels for the same application has an added advantage since one of the major issues faced with currently available skin substitutes is the high manufacturing and handling costs. With the use of GelMA in skin substitutes, the cost of materials can be reduced. Furthermore, the adjustment of the various parameters during GelMA synthesis and crosslinking allows regulation of the final mechanical and chemical properties of the resulting hydrogel. This makes GelMA flexible and easy to work with.

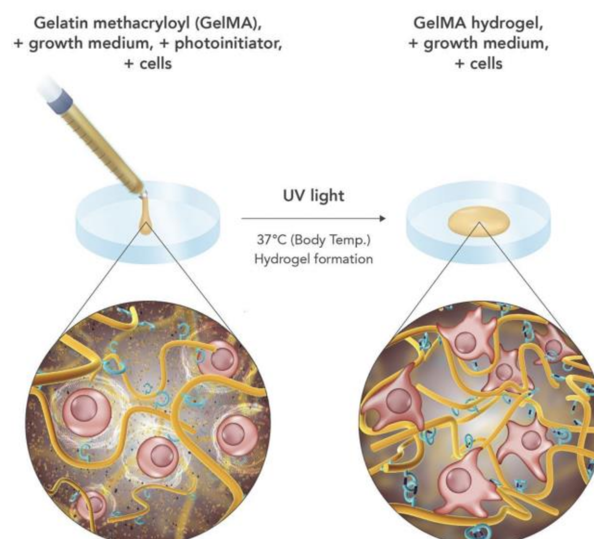

Figure 4: Cell activity in GelMA.

3.3. PREPARATION OF GelMA:

The preparation and synthesis of GelMA involves a number of chemical processes such as dissolution, dialysis and freeze drying. 5 grams of gelatin was dissolved in 50ml Dulbecco's Phosphate Buffered Saline (PBS) under constant stirring of 300 rpm at 50 degree celsius using a water bath, until fully dissolved leaving no residue. Once the gelatin was fully dissolved, 5ml (1ml for every gram of gelatin) of MA was added to the solution dropwise, also under constant stirring at 50 degree celsius, using a dropper. The entire apparatus was covered in aluminium foil to avoid the solution from interacting with light due to the light sensitive nature of MA. After all the 5ml of MA had been dissolved into the solution containing gelatin and PBS, it was set aside in a 50 degree celsius water bath for 3 hours. The degrees of substitution of GelMA can be changed by varying the volume of MA which affects the porosity, the compressive modulus and swelling behaviour of the hydrogel.
At the end of the 3 hours, 200ml of PBS was added to the solution at 40 degree celsius and set aside for 2 hours. On the completion of the 2 hours, the solution was ready for the process of dialysis (Zhu et al., 2019). Dialysis was done by separating the gel into 4 different dialysis membranes of 12-14Da and immersing them into deionised water under 40 degree celsius under constant stirring. This was also done in an apparatus covered with aluminium foil to avoid cross linking of the gel solution on interaction with light. Dialysis was carried out for 7 days and the deionised water was changed once in every 3 hours. The aim of the dialysis process was to remove poisonous and unreacted MA as well as other by products. The dialysis of GelMA solution against water leads to a flexible, transparent gel. The last step before obtaining GelMA in its dry state is to freeze dry the dialysed solution for 7 days. Freeze-drying removes moisture from raw, frozen GelMA through a vacuum system using the process of sublimation. At the end of 7 days, a spongy white residue is left back. The residue is GelMA in its dry state. This can then be dissolved in the required amount of PBS under 50 degree celsius to obtain GelMA in its original gel form after which a photo initiator is added to cross link the gel using UV light (figure 5).

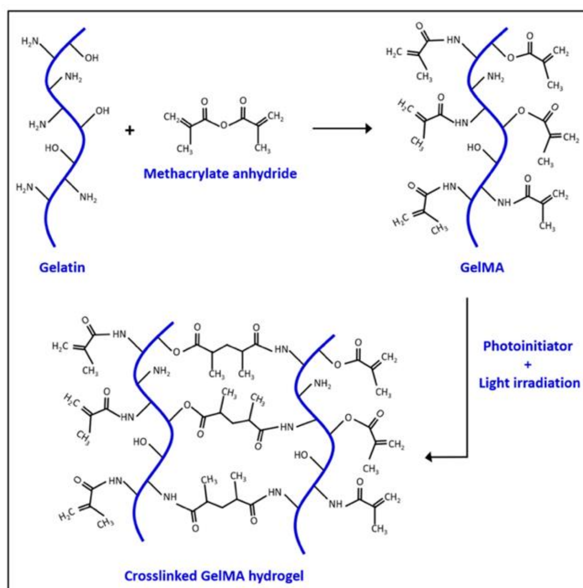

Figure 5: GelMA synthesis and cross linking

3.4. MODIFICATION OF GELMA:

a. Concentration of GelMA:

The varying concentrations of GelMA in PBS show different properties of the gel. A solution of 2% GelMA in PBS was prepared to check for gelation. Although 2% is the usual concentration that is used for most gels it was seen that for GelMA there was not the case as there was no gel formation. However, on increasing the concentration to 5% it was seen that there was homogenous gel formation and that the gelation was retained on immersing it in water at 50 degree celsius. Therefore, 5% GelMA was made the standard for the rest of the experiments.

b. Photo Initiator:

Both UV and visible light can initiate photo crosslinking of GelMA up to a certain extent although the most common photo initiators that are currently used work within the UV range. The UV light irradiation parameters can be adjusted and varied to investigate the mechanism of crosslinking. The photo initiator used for this experiment is Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP). Different concentrations of the photo initiator were used to obtain an ideally crosslinked gel (Xiao et al., 2019). A concentration of 0.1% (weight/volume) was used initially and then gradually increased up to 0.25% at which it was seen that the gel crosslinked best, with UV irradiation. At this concentration, the photo initiator was seen to be non-toxic to the cells, however at higher concentrations it could cause toxicity. The gel was then crosslinked by irradiating it with UV light of 405nm for a period of 20 seconds, 40 seconds, 60 seconds and 2.5 minutes after which its rheological properties were studied.

3.5. RHEOLOGY TESTS:

A solution of 5% GelMA in PBS along with 0.25% (weight/volume) photo initiator was made by dissolving the GelMA in PBS at 50 degree celsius and constant stirring after which the photo initiator was added in the completely dissolved solution. The gel was then crosslinked and tested for rheological properties. The UV light used for cross linking the gel was of wavelength 405nm. 405nm was chosen as this wavelength does not cause harm to the gel nor cause toxicity to the cells. The time of exposure to UV light was varied from 20 seconds, 40 seconds, 60 seconds to 2.5 minutes. For each time interval the gel was run through three tests in order to check for reproducibility and consistency in the results. 200 micro litre of the gel was pipetted out and dropped into a circular mould having almost the same diameter of the geometry of the rheometer. The gel was then irradiated with UV light for 20 seconds directly from above the sample from a height of 5cm. This was done on a separate surface and not on the rheometer. Irradiation with UV light led to cross linking of the gel after which it was transferred to the rheometer and the test was carried out under 37 degree celsius (human body temperature). A frequency graph was plotted for each of the three repetitive tests and compared for consistent results. The same was then repeated for irradiation times of 40 seconds, 60 seconds and 2.5 minutes. All the tests were carried out in a dark environment to avoid cross linking of the gel by means other than that of UV light.

a. RHEOLOGY TEST RESULTS:

On crosslinking the gels by irradiation with UV light of 405nm for varying time intervals it was seen that the gel that was irradiated for 40 seconds showed the best results. Gelation was clearly visible, even to the naked eye. The gel showed consistent results for all three repetitions and was neither too stiff nor too soft for cell growth and activity and for use

in cell culture experiments. Irradiation for 20s showed consistent results however the cross linked gel was unsuitable for cell culture experiments. GelMA cross-links completely after a minimum of 20 seconds of UV exposure, which results in the gel being too soft for cell growth. Irradiation of the gel by UV light for 60 seconds resulted in a cross linked gel similar to the one obtained with 40 seconds, however it was seen that the gel was stiff and may cause toxicity to the cells in the long run due to increased exposure to UV light which was the same reason for the gel that was cross linked for 2.5 minutes to be excluded from use. Longer exposure to UV light is toxic for cell growth in the long run and 2.5 minutes of exposure to UV light causes the gel to become too stiff for cell spreading (Zhu et al., 2019) [17]. It was also seen that when the lower end amount of the photo initiator was used, the cross-linking time takes significantly longer which leads to toxicity but increasing the concentration of the photo initiator also could be toxic to cells. Hence it is important to optimise the concentration of the photo initiator and the UV irradiation time.

The results of all four cross linking times were plotted in a simple frequency dot plot (Figure 6) with angular frequency against elastic modulus(G') and loss modulus(G"). Elastic modulus is the ratio of shear stress to shear strain that the gel represents. Loss modulus measures the heat energy dissipated when the gel starts to turn viscous.

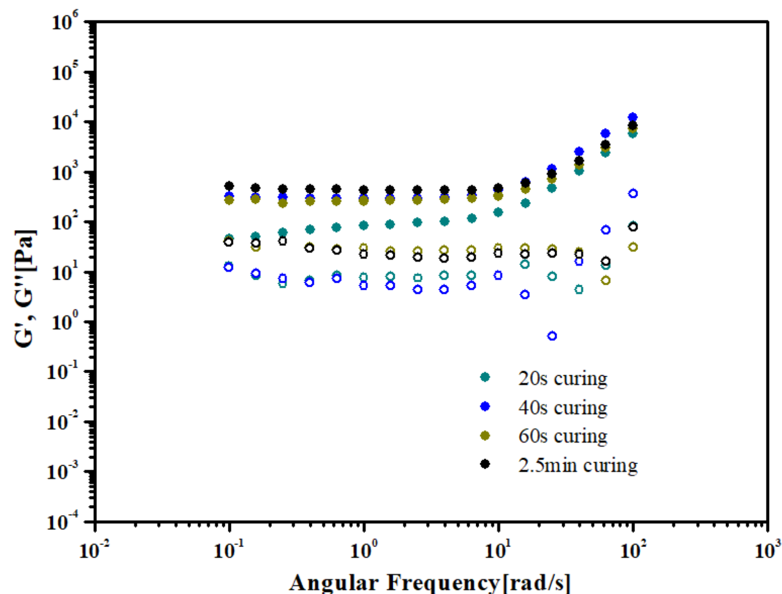

Figure 6: Rheology test results.

3.6. STERILIZATION AND 3D CELL CULTURE:

Sterilization of the gel before use in cell culture experiments is a key step to maintain cell safety and health in tissue engineering. The removal of all microorganisms, including bacteria and fungi is essential to attain successful initiation, spreading, growth and development of the cultured cells in vitro, which otherwise would be overwhelmed by the foreign material leading to contamination and cell death (Rizwan et al., 2020) . The methods of sterilization used here are UV sterilization and filtration. First, GelMA in its dry state is exposed to UV light for 2 hours for disinfection and killing of any prominent viruses. In the next step the solution containing 5% GelMA in PBS is prepared under constant heat (50 degree celsius) and stirring. This gel is then filtered using cell culture filter units to filter out any bacteria and precipitation using its small pore size. Once the gel was filtered, it was

incorporated into petri dishes and cross linked for 20 seconds, 40 seconds and 60 seconds. The cells were then allowed to grow in the same petri dishes along with the gel and cell medium. The cell lines that were used for the experiments was human primary dermal fibroblast of $1x10^6$cells/ml in 0.5ml/well.

a. 3D CELL CULTURE RESULTS:

Cells were cultured in 5% GelMA with 5mg photo initiator in 2ml PBS and imaged using an optical microscope after being incubated at 37 degree celsius for a period of 1 day and 3 days. The cells were stained with F-actin and nuclei with phalloindin/DAPI. These stains are used to visualise nuclear DNA in both living and fixed cells. It was seen that the cells that were cultured in the GelMA that was crosslinked for 40 seconds had started to flatten out and showed the best cell spreading behaviours after 3 days compared to those that were cultured in the gel that was cross linked for 20 and 60 seconds (figure 7).

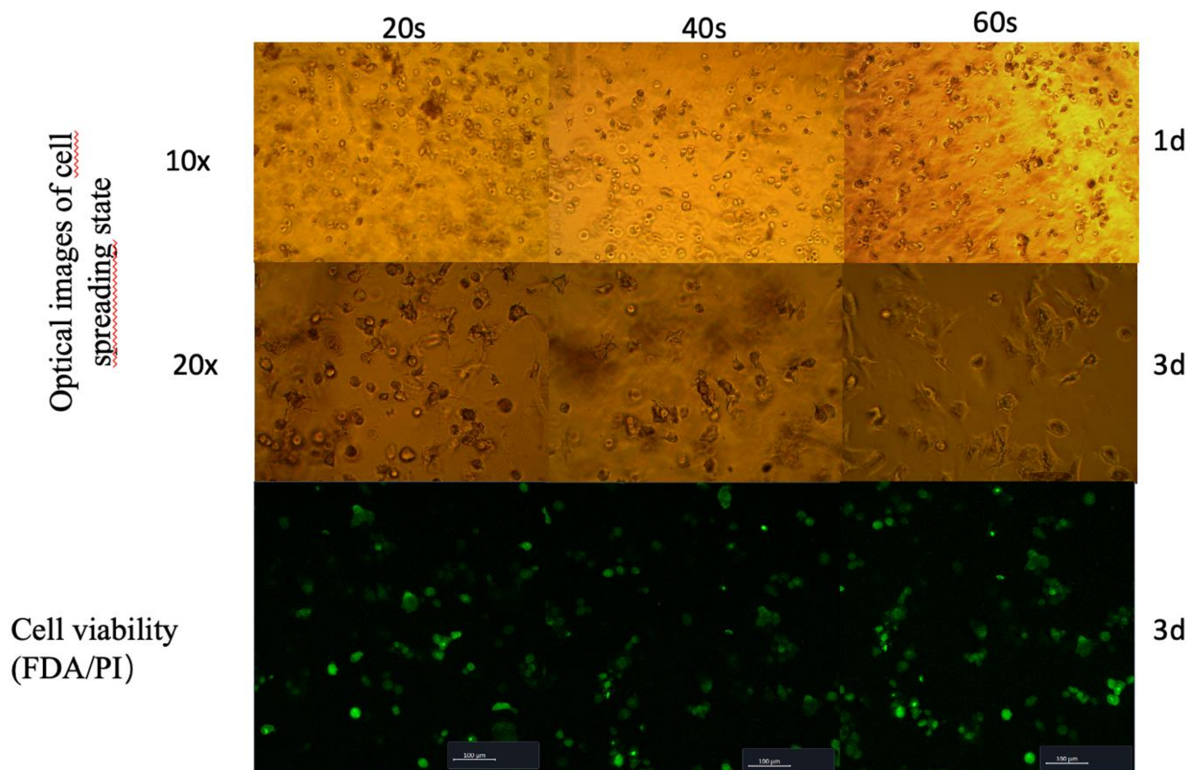

Figure 7: 3D cell culture showing cell spreading and viability for 1 and 3 days. (Image courtesy: Xixi Wu, May 2022)

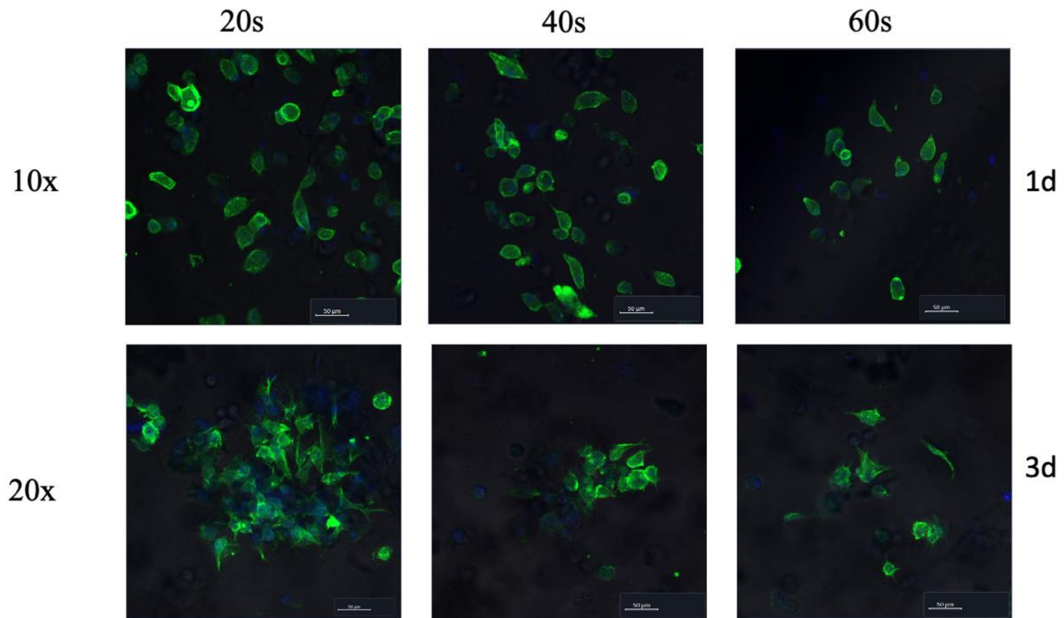

Figure 8: Cell spreading behaviour as seen after 1 and 3 days of cell culture.
(Image courtesy: Xixi Wu, May 2022)

The cells were also cultured for a period of 7 days to check for viability. Here, cells were cultured in 5% GelMA with 5mg photo initiator in 2ml PBS and imaged using an optical microscope after being incubated at 37 degree celsius for a period of 7 days. Here FDA/PI were used to stain the cells to check for the enzyme activity of the cell population. However, here the cell behaviour was better when the gel was irradiated for 20 seconds by UV (figure 9). This behaviour was contrary to the behaviour shown in the 3 day cell culture where 40 second irradiation showed better results. From this it was inferred that longer time of cell culture showed cytotoxicity of longer irradiation from the UV light.

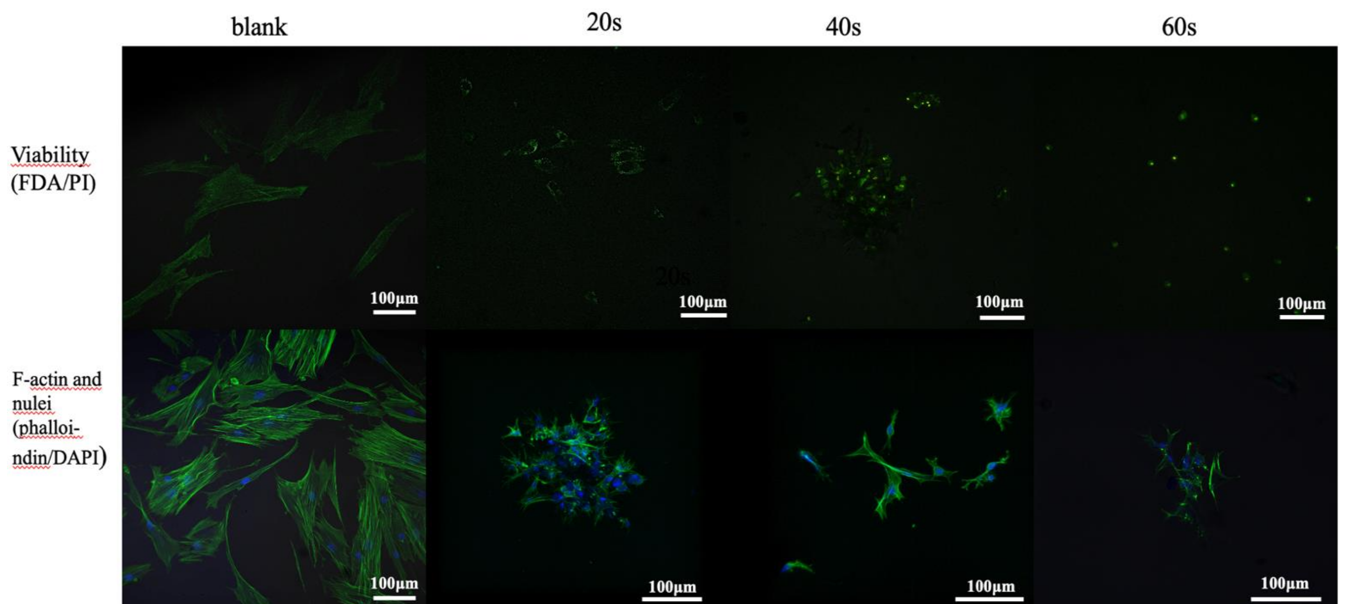

Figure 9: 7 day cell culture results and cell behaviour
(Image courtesy: Xixi Wu, June, 2022)

3.7. DEGRADATION TESTING:

Degradation is a critical property of gels used in tissue engineering applications. Biodegradable scaffolds are preferred because in the tissue reconstruction process initially the cells are grown on the scaffolds and then penetrate the structure and in the meantime, the scaffold degrades creating more space for growth of the cell. To study the degradation property of GelMA, photo cross linked samples that were irradiated for 20, 40 and 60 seconds and 2.5 minutes were weighed and the weight of each sample was recorded separately. Degradation testing was done under room temperature initially for which PBS was added in the samples. They were weighed after a time period of 0 hours, 7 days and 14 days and the results were recorded. There was not much mass difference observed in degradation under room temperature (figure 10). Since the gel was going to be used in the human body for skin substitutes, the degradation testing was also carried out under 37 degree celsius with all the other parameters remaining the same. This time, the gel also had PBS in it. The results were recorded after 0 hours and 7 days. Again, there was not a significant mass difference, however, there was almost a 0.0010g difference in mass for the different time intervals (figure 11).

Curing Time Weight(g) Degradation Period	20s	40s	60s	2.5min
0h	0.0125	0.0119	0.0105	0.0102
7d	0.0122	0.0120	0.0125	0.0154
14d	0.0159	0.0152	0.0151	0.0115

Figure 10: Degradation testing under room temperature.

Curing Time Weight(g) Degradation Period	20s	40s	60s
0h	0.0145	0.0139	0.0131
7d	0.0152	0.0146	0.0149

Figure 11: Degradation testing under 37 degree celsius.

4. RESULTS:

The experiments that were conducted throughout the research showed proof that GelMA is indeed an ideal hydrogel for cell culture experiments and could potentially be used to develop skin substitutes. However, the gel had to be optimised for this application. 5% GelMA in 2ml PBS along with 0.25% photo initiator proved to show the optimum results for cell growth and activity. Cross linking time of 40 seconds showed good cell spreading and viability in the short term (3 days), however, in the long run turned out to be toxic to the cells due to elongated time of UV irradiation (Pepelanova et al., 2018). This is where the cross linking time of 20 seconds proved to be efficient since cell spreading and viability was seen after 7 days of cell culture (figure 8). The cross linking time of 60 seconds and 2.5 minutes turned out to make the gel too stiff for cell culture experiments and could also be toxic due to elongated time of UV exposure. Degradation experiments showed that the gel did not show a great variance in mass loss from 0 hours to 7 days under body temperature. However, this needs to be further studied and modified to obtain more accurate results.

5. DISCUSSIONS AND CONCLUSIONS:

Tissue engineered skin substitute preparation involves cells and extracellular matrix. An ideal synthesized skin substitute should be sterile, act like a barrier and provide no toxicity. They must also have required physical and mechanical properties and go through controlled degradation. Easy availability, long-shelf life and cost effectiveness makes an ideal engineered substitute. A suitable hydrogel is one of the main components in the development of functional skin substitutes. GelMA satisfies most of the above requirements and hence could make a suitable hydrogel for 3D cell culture and skin substitute applications. The adjustment of the various parameters of GelMA synthesis allows regulation of the final mechanical and chemical properties of the resulting hydrogel. The experiments conducted in this research proved the same. The process of synthesis of GelMA yielded good results throughout the research and hence was a success. However, results of the 3D cell culture experiments showed that the cells that were cultured in gel that was irradiated with UV light for 40 seconds in the 7 day cell culture showed lesser cell spreading and viability as compared to the same group in the 3 day cell culture. It was inferred that the longer time of UV exposure might have caused the cell toxicity but there is no proof that this is the only cause for cell death. This needs to be further studied and assessed to understand the other factors that may have caused the cells to proliferate lesser after 7 days. This might alter the future direction of the research. The degradation testing under body temperature showed a difference in weight of about 0.0010grams in each of the three groups but no conclusion can be made out for this since the 14 day sample was not weighed due to time restraints. However, this could be completed in the future work of the research. Compressive tests need to be carried out to support the gels use in skin substitutes. Further research and development is required to better optimise GelMA for cell culture applications and for use in skin substitutes. Overall, it can be concluded that the basic goal of the research was met. The development and synthesis of GelMA was most successfully completed, although it remains unclear if the accuracy level requirement is met. As previously mentioned, more research is required to determine if these requirements are fulfilled. However, not meeting the accurate aims of the research is not an issue as it will help to determine which parts the research process should be improved to allow to gain more insight on future perspectives of the study.

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