3D Scaffolds in the Treatment of Diabetic Foot Ulcers: New Trends vs Conventional Approaches

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3D Scaffolds in the Treatment of Diabetic Foot Ulcers: New Trends vs Conventional Approaches

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Abstract: Diabetic foot ulcer (DFU) is a serious complication of diabetes mellitus, affecting roughly 25% of diabetic patients and resulting in lower limb amputation in over 70% of known cases. In addition to the devastating physiological consequences of DFU and its impact on patient quality of life, DFU has significant clinical and economic implications. Various traditional therapies are implemented to effectively treat DFU. However, emerging technologies such as bioprinting and electrospinning, present an exciting opportunity to improve current treatment strategies through the development of 3D scaffolds, by overcoming the limitations of current wound healing strategies. This review provides a summary on (i) current prevention and treatment strategies available for DFU; (ii) methods of fabrication of 3D scaffolds relevant for this condition; (iii) suitable materials and commonly used molecules for the treatment of DFU; and (iv) future directions offered by emerging technologies.

Keywords: additive manufacturing; bioprinting; cold-plasma; diabetic foot ulcers; emerging technologies; electrospinning; implants

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63 1. Introduction

64 Diabetes mellitus (DM) is among the top ten causes of deaths worldwide and places a
65 significant demand on healthcare systems (World Health Organisation, 2020). The term DM
66 encompasses two subtypes, including type 1 diabetes (T1DM) and type 2 diabetes (T2DM).
67 T2DM accounts for approximately 90% of global diabetes cases, while T1DM is less common
68 and accounts for approximately 5 - 10% of global cases (Kahanovitz et al., 2017). The
69 pathophysiology of T1DM and T2DM differ; however, both are underpinned by the failure of
70 the body to process and utilise glucose correctly, resulting in raised levels of glucose circulating

in the bloodstream. Currently, 463 million people are estimated to suffer from DM worldwide (International Diabetes Federation, 2020). The incidence of DM is increasing rapidly worldwide, primarily due to various social and economic factors, with a predicted 700 million people to be affected by 2045 (International Diabetes Federation, 2020).

The rising incidence of DM has huge economic implications. In the US alone, \$327 billion is spent annually on both medical costs and as a result of lost work days due to DM (Centre of Disease Control and Prevention, 2020). In the UK, an estimated £837 million and £962 million were spent on DFU treatment and associated amputations between 2014 and 2015 (Kerr et al., 2019).

One major complication of DM is the development of DFU. For diabetic patients, a simple tear of the skin can have serious consequences. Up to 25% of patients suffering from DM develop DFU, with over 70% of these patients requiring lower limb amputation (Peter-Riesch, 2016). The DFU market alone is set to increase from \$7.03 billion USD in 2019 to \$11.05 billion USD by 2027 (Fortune Business Insights, 2019).

Several factors contribute to increase in incidence of DFU, such as inadequate wound healing, which is frequently observed in diabetic patients, and will be discussed in this review. Due to a rapid increase in DM incidence worldwide, a correlating increase in the occurrence of DFU has been predicted, as well as increasing healthcare costs and resultant amputations in the absence of effective treatment options, which is supported by current market predictions (Fortune Business Insights, 2019).

1.1.Normal wound healing process

Normal wound healing is a complex, interwoven process consisting of multiple stages, which can occur sequentially or simultaneously in different sections of the wound. Coagulation, inflammation, proliferation and wound remodelling contribute to an overall effective and complete wound healing process in healthy individuals. This is achieved through a tightly regulated and co-ordinated response. Numerous cells, cytokines and growth factors mediate this process to promote appropriate conditions for complete tissue repair in each stage of the wound healing process.

In the normal wound healing process, haemostasis occurs immediately following injury. Vasoconstriction of blood vessels surrounding the site of injury is facilitated by upregulation

of thromboxane's and prostaglandins and temporarily acts to prevent excessive blood loss. Platelets bind to exposed collagen and initiate the coagulation cascade, which results in the formation of a fibrin clot, *via* enzymatic conversion from fibrinogen. Circulating platelets then release platelet-derived growth factor (PDGR) and transforming growth factor (TGF), signifying progression to the inflammatory stage (Pierce et al., 1991).

Leukocyte rolling causes neutrophil migration to the site of injury, facilitated by the upregulation of integrin receptors on the endothelial surfaces of blood vessels. Leukocytes act to phagocytose debris, secrete reactive oxygen species (ROS) and pro-inflammatory cytokines (Ellis et al., 2018, Wilgus et al., 2013). Next, monocytes move to the site of injury, which differentiate into the pro-inflammatory M1 phenotype macrophages to allow the release of pro-inflammatory cytokines, such as IL-1 and TNF- α (Olingy et al., 2017). This encourages lymphocyte migration to the site of injury. The polarization of macrophages to the anti-inflammatory M2 phenotype, and subsequent release of factors such as vascular endothelial growth factor (VEGF), then signals progression towards the third stage of wound healing. The proliferative stage of wound healing involves fibroblast migration, collagen synthesis, angiogenesis, granulation tissue formation and epithelialisation. Deposition of extracellular matrix (ECM) to replace the temporary fibrin clot occurs during the remodelling phase (Petreaca and Martins-Green, 2020), which is laid down by fibroblasts and provides structural robustness to the wound.

1.2. Diabetic wound healing process

Several factors throughout the wound healing process contribute to the abnormal and incomplete wound healing often observed in diabetic patients, as summarised in Fig.1. Most prominently, continued inflammation has been shown to lead to poor wound healing outcomes. Where the normal wound healing process is typically complete in 4 – 6 weeks (Wallace et al., 2020), diabetic wound healing often extends beyond this timeline and is often left unresolved, leading to infection and amputation in the majority of cases (Peter-Riesch, 2016).

Dysregulation of both monocytes and macrophages plays a role in the abnormal diabetic wound healing response. An observed inability of macrophages to polarise from the pro-inflammatory M1 phenotype to the anti-inflammatory M2 phenotype following the inflammatory stage of wound healing leads to prolonged inflammation, contributing to incomplete wound healing. In addition, greater expression of monocytes as a result of modifications to hematopoietic stem

and progenitor cells (HSPC) leads to poor wound healing and impaired phagocytosis due to overexpression of macrophages, in particular the M1 phenotype (Barman and Koh, 2020).

Other key cells involved in wound healing, such as neutrophils, have been shown to be negatively affected by the physiological conditions created by DM. Increased levels of neutrophils, as well as neutrophil extracellular traps (NETs), are known to contribute to poor wound healing in diabetic patients (Yang et al., 2020). One study found treatment with dapsone, an antibacterial agent commonly used in the treatment of skin diseases such as dermatitis, in an *in vivo* diabetic rat model led to improved wound healing when compared to the control group, indicating the positive effect of IL-8 inhibition and the resultant reduction in neutrophil levels during diabetic wound healing. High level of expression of other inflammatory markers such as inducible nitric oxide synthase (iNOS), IL-12 and TNF- α , while reduced expression of anti-inflammatory markers such as IL-4 and IL-10, are indicative of a prolonged inflammatory response characteristic to chronic wounds (Ellis et al., 2018).

Moreover, studies have found transcription factors Forkhead Box M1 (FOXM1) and signal transducer and activator of transcription 3 (STAT3), which facilitate tissue repair *via* activation and recruitment of neutrophils and macrophages, to be reduced in diabetic wound healing (Sawaya et al., 2020).

Nitrogen oxide (NO) synthesis occurs primarily during the proliferative wound healing stage and in short facilitates wound healing *via* collagen formation and cell proliferation of key cells such as macrophages and keratinocytes (Malone-Povolny et al., 2019). In diabetic patients, the inhibition of nitric oxide synthase leads to a reduction in NO and increased superoxide, as well as other reactive species. Increased production of advanced glycation end products (AGEs) as a result of hyperglycaemia has been reported to contribute to increased levels of ROS and reactive nitrogen species (RNS), which can cause significant tissue damage (Malone-Povolny et al., 2019). Overall, a reduction in antioxidants due to reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH) and increased ROS and oxidative stress impel the complications of DFU.

In combination, these factors contribute to the overall impaired wound healing response observed in diabetic patients.

1.3. Pathology of DFU

Several underlying pathological risk factors contribute to the development and abnormal healing of DFUs; however, the most prominent of these include diabetic neuropathy (DN), peripheral arterial disease (PAD) and immunosuppression.

An estimated 20% of T1DM and 50% of T2DM suffer from DN (Pop-Busui et al., 2017). Clinically, loss of sensation due to peripheral nerve damage means DFU can go overlooked for prolonged periods in the absence of regular foot check-ups. This allows ulcer development to progress unnoticed. During this time, the wound may be subjected to continuous pressure over prolonged periods of time, or to further physical damage due to loss of sensation, leading to more severe ulceration. The nerve damage observed in the intrinsic foot muscles of diabetic patients results in imbalance of flexion and extension, ultimately creating pressure points for where ulceration can occur (Clayton and Elasy, 2009).

Peripheral arterial neuropathy (PAD) in diabetic patients is thought to result primarily from the two-step reaction polyol pathway. This metabolic pathway accounts for 30% of glucose consumption but is implicated in diabetic patients through upregulation in response to elevated glucose levels (Yan, 2018). In this pathway, glucose is firstly reduced to sorbitol by aldose reductase enzyme, followed by conversion of sorbitol to fructose by sorbitol dehydrogenase. Under normal physiological conditions this process results in the production of nicotinamide adenine dinucleotide + hydrogen (NADH) via NADPH consumption; however, in diabetic patients redox imbalance occurs due to increased levels of NADH (Wu et al., 2016). As a result, increased superoxide's, a precursor to ROS, are produced due to enhanced electron transportation via Complex 1. The oxidative stress as a result of enhanced NADH levels has been noted as a major contributor to DN (Mallet et al., 2020). Furthermore, in diabetic patients the upregulation of the polyol pathway results in elevated sorbitol levels in the nerves, contributing to reduced myo-inositol levels, $\text{a+}, \text{K}^+$ -ATPase activity and reduced nerve functionality (Nesterova et al., 2020).

There is an increasing need for further insight into combination therapies for pain relief, as well as long-term efficacy studies, on diabetic patients suffering from DN. Moreover, there is a need for advanced drug delivery systems to improve the treatment of DN, reflected by a predicted increase in DN market growth to meet patient demand. In North America, the DN market size is predicted to reach \$7.12 billion by 2026 (Fortune Business Insights, 2018).

Neuropathy experienced by diabetic patients is not limited to DN. Autonomic neuropathy, caused by sweat gland dysfunction, is a common manifestation of DM and results in the inability of the body to moisturise the skin, leaving open cracks (Volmer-Thole and Lobmann, 2016). This can initiate DFU development while also providing an entry route for pathogens due to disruption of the physical protective barrier that is provided by the skin.

Once the DFU has been identified and treatment has been initiated, PAD commonly impedes the process of normal wound healing. It does so through several mechanisms and contributes to ulcer progression in up to 50% of cases (Clayton and Elasy, 2009). Endothelial cell dysfunction caused by hyperglycaemia reduces the production of endothelial-derived vasodilators, such as NO and prostacyclin (Avogaro et al., 2011), potentially increasing the risk of atherosclerosis occurring.

Furthermore, an increase in the vasoconstrictor thromboxane A₂ has been observed in diabetic patients, which plays a role in enhanced platelet aggregation (Santilli et al., 2020). Disruption of the microcirculation also occurs following arteriolar-venular shunting. Overall, this leads to ischemia at a time where enhanced blood flow is required to facilitate the normal wound healing process. Ischemia is observed in approximately half of diabetic patients and is a factor highly associated with lower limb amputations (Shatnawi et al., 2018).

Immune factors also play a role in the abnormal wound healing response observed in diabetic patients. Many of these patients have impaired immune function, meaning cells, which are key to a normal wound healing response are not activated or functioning properly. In addition to this, the risk of infection for these patients is higher and could act to further slow the wound healing process due to infection or biofilm development. For example, *Enterococcus faecalis*, a microorganism commonly associated with DFU infection, has been found to impair the wound healing response at high concentrations (Chong et al., 2017).

Several reviews have provided in detail insight into the underlying pathological risk factors and physiological factors which lead to DFU development and progression (Bandyk, 2018; Mishra et al., 2001; Patel et al., 2019)

1.4. Diabetic foot ulcer classifications

Various classification systems have been established to indicate disease progression. Most importantly, the cause of the ulcer and whether it is neuropathic, neuro-ischemic or ischemic should be evaluated in order to predict DFU outcome (Alavi et al., 2014).

The most commonly used classification system for DFU is the Wagner Ulcer Classification System (WUCS). This system classifies ulcers based on ulcer depth and presence of osteomyelitis or gangrene (Swezey, 2016); however, does not address ischemia nor infection. The classification scale ranges from 0 to 5, moving from intact skin to whole foot gangrene with increasing classification numbers (Swezey, 2016).

The University of Texas Diabetic Foot Ulcer Classification System is another commonly used system. Unlike WUCS, DFU are classified based on the occurrence of ischemia or infection as well as ulcer depth. Grades 0 to 3 range from healed pre- or post-ulcerative sites to a wound that penetrates the bone or joint, respectfully. Within each Grade, four further subcategories exist to assess infection and ischemia parameters, with Stage A representing clean, non-infectious wounds while stage D represents ischemic infectious wounds (Swezey, 2016).

According to literature, the Perfusion Extent Depth Infection and Sensation (PEDIS) classification system provides excellent validity and reliability in predicting DFU outcome, based on the parameters described in the classification title (Chuan et al., 2015). Combination of grade and score, based on each of the five categories, results in a minimum score of one and maximum of 12, indicating on overall severity and prognosis (Chuan et al., 2015). It is more objective and faces stricter criteria than previous methods such as WUCS.

The Site Ischemia Neuropathy Bacterial Infection and Depth (SINBAD) system provides the tools to make predictions regarding ulcer outcome and how this varies between countries, based on a score between 0 and 6 (Arteri-, 2008). Recently, SINBAD was found to have similar accuracy and reliability in predicting DFU outcomes as the University of Texas classification system (Leese et al., 2020).

Diabetic Ulcer Severity Score (DUSS) is used to evaluate parameters such as “palpable pedal pulses, probing to bone, ulcer location, and presence of multiple ulcerations”. This method has been shown to accurately predict disease progression, based on implementing a scale of 0 to 4 of increasing clinical severity, in a standardised manner (Beckert et al., 2006).

Recent analysis into the accuracy and reliability of WUCS, SINBAD, UT and PEDIS concluded, that in order to ensure an accurate clinical assessment, these classification systems should be used in combination. While WUCS provided the most accurate and reliable predictions of ulcer outcome, PEDIS showed the least reliable outcomes, with moderate. Meanwhile, SINBAD and UT both showed good reliability (Camilleri, 2019).

2. Current strategies used to enhance wound healing

As with many diseases, early implementation of treatment is one key aspect to a successful treatment outcome for DFU healing. Wound healing strategies can fall under standard therapies or advanced therapies. As ulcer severity progresses, a transition from standard therapies, such as wound dressings, to incorporate advanced therapies, such as negative pressure wound therapy (NPWT), may be necessary to achieve successful healing outcomes. Current wound healing strategies for DFU are summarised in Table 1.

2.1. Advance therapies

Commonly used advanced therapies in the treatment of DFU have been summarised in Table 2.

2.1.1. Offloading/pressure control

Foot ulceration in diabetic patients commonly occurs due to physical damage from the outside environment. This damage may be a result of a short-term, high pressure in a small focused area or prolonged low pressure in a more widespread area. This stress on select areas of the foot can often result in ulceration, which can be further complicated by the presence of peripheral arterial disease and diabetic neuropathy. The nerve damage, which occurs due to DN can affect the patient's gait and therefore lead to abnormal cadence, focusing pressure on select of areas of the foot and making the patient susceptible to ulcer formation.

As DFU have been shown to occur in areas of high plantar pressure, this highlights the important initial role offloading plays in the treatment of DFU (Fernando et al., 2016). Various methods for offloading include crutches, surgical shoes and bandages (Mishra et al., 2001).

Comprised of specialised materials, total contact casts (TTCs) have been referred to as the gold standard for offloading when it comes to DFU treatment. For the management of DFU, the International Working Group of the Diabetic Foot (IWGDF) recommend a non-removable

knee-high offloading device with appropriate foot-device interface as the primary treatment option for neuropathic plantar or midfoot DFU. Other treatment options for offloading include a removable knee-high device, removable ankle-high device or felted foam to be placed as cushioning in appropriate footwear (Bus et al., 2020).

As outlined in Table 1, TTCs have been shown to greatly reduce the time for DFU healing when compared to conventional dressings (Sahu et al., 2018; Sudhir et al., 2020). Non-removable devices have been found to be more effective than removable devices (Morona et al., 2013). These may be owed to their benefits such as improved patient compliance.

There are limited risks associated with the use of TTCs. Due to immobilisation of the limb the risk of complications such as venous thrombo-embolism (VTE) would be expected to increase. A study into this matter did not find any occurrence; however, larger study samples are needed to confirm this finding (King et al., 2017). A ten-year study found very low prevalence of VTE, providing long-term insight into this potential complication associated with TTC use, but requires the same duration of insight using larger sample sizes to gain reliable conclusions on this matter. Risk of occurrence should be evaluated on an individual basis, taking into account underlying conditions (Tonge et al., 2019).

2.1.2. Glycaemic control

As previously outlined, prolonged hyperglycaemia in diabetic patients can lead to DN and PAD, both of which have a negative influence on DFU healing. Glycaemic control therefore has to be achieved to promote successful wound healing.

Elevated levels of HbA1C, a glycated haemoglobin, show average blood sugar levels over a two to three month period (Diabetes UK, 2020) and can act as an indicator of poor glycaemic control.

Variability in healing time of DFU has been associated with varying levels of glycaemic control in patients, with lower HbA1C levels associated with faster healing rates (Dhatariya et al., 2018). As shown in Table 1, another study found a HbA1C level between 7 – 8% would provide benefit to the wound healing of DFU (Xiang et al., 2019). Negative correlation has been reported between 1.0% increases in HbA1C levels and the rate of DFU wound healing (Christman et al., 2011). In paradox to these findings, one study concluded that an increase in HbA1C has been associated with improved wound healing in patients where baseline HbA1C

was below 7.5%, which may have resulted from unidentified hypoglycaemia (Fesseha et al., 2018). Furthermore, reduced glycaemic levels have been found to improve wound healing but do not improve neuropathy or vasculopathy (Kumar et al., 2016).

2.1.3. Debridement

Debridement is the removal of necrotic and senescent tissues as well as foreign and infected materials from a wound (Healthline, 2019). This is generally the first step of DFU treatment and can be performed through various methods, such as surgical, mechanical, enzymatic, autolytic and biological. These methods can be used in isolation or in combination. Despite the chosen method, the overall aim of debridement is to promote wound healing through the stimulation of a chronic wound in order to create an acute wound and allow rapid, effective healing.

Ultrasound assisted debridement has also shown promising and rapid results in terms of reducing bioburden and promoting wound healing of neuro-ischemic DFU (Lázaro-Martínez et al., 2018). This method of debridement has also been shown to reduce debridement times and potential complications (Campitiello et al., 2018).

Surgical debridement involves removal of both non-viable tissue and immediately surrounding viable tissue in order to stimulate wound healing, and thus is suitable for when large areas of tissue must be removed. Surgical debridement has been found to not be necessary for patients with sufficient peripheral arterial perfusions, which could help implement the use of effective treatments such as photodynamic therapy sooner and thus reduce treatment times (Tardivo et al., 2017).

Similar to surgical debridement, sharp debridement is a cost-effective method given the limited equipment required and absence of anaesthetic; however, requires skilled personnel in order to prevent accidental enlargement of the wound area, which could reduce wound healing capabilities.

In terms of biological debridement methods, the use of maggot debridement therapy (MDT) has proven successful. MDT accelerates the rate of wound healing while also demonstrating antiseptic abilities and stimulate granulation tissue formation (Azad et al., 2016). This method is cost effective and these genetically engineered maggots can be used to secrete human platelet derived growth factor BB (PDGF-BB) (Linger et al., 2016).

Autolytic debridement is the lysis, or breakdown, of damaged tissue at a wound site by the body's natural defence system by enzymes that digest specific components of body tissues or cells, for example fibrin and collagen (Choo et al., 2019). This method is highly selective, has little risk for complications or side effects and is suitable for small areas, but is a slow method of debridement (Manna et al., 2020).

The process of mechanical debridement involves the application of a wet gauze which then adheres to the skin and, upon its removal, results in the removal of dead tissue surrounding the wound (Ananian, 2020). This method is non-selective and requires frequent dressing changes, as well as causing pain and bleeding in some instances (Wound Source, 2019).

Enzymatic debridement makes use of natural proteolytic enzymes to remove non-viable tissue in a selective manner. Collagenase products, papain-based products and papain-urea-chlorophyllin complex have all been used for this method. Neuropathic non-ischemic DFU treated with clostridial collagenase ointment (CCO) witnessed improved wound closure when compared to the daily hydrogel treatment group following 6 weeks of treatment (Jimenez et al., 2017). Using CCO in combination with sharp debridement has proven to have both clinical and economic benefits (Motley et al., 2015).

2.1.4. Infection management

In many instances, insufficient wound healing and amputation is preceded by the presence of pathogenic microorganisms in the ulcerated area. This is due to several factors, including the prolonged exposure of the wound area as a result of reduced healing times in diabetic patients. A study by Jia et al., suggested infections of DFU to have a prevalence of approximately 40% (Jia et al., 2017); however, earlier reports have suggested this figure to be as high as approximately 60% (Prompers et al., 2007).

Firstly, the pathogen must be identified to ensure appropriate treatment, as the recommended treatment duration will vary between pathogens. According to the IWGDF, the severity of infection should be classified using the classification scheme proposed by Infectious Diseases Society of America/International Working Group on the Diabetic Foot (Bus et al., 2019). Identification of the pathogen involves obtaining a soft tissue or bone sample for microbiological analysis.

Once identified, infection management is controlled primarily through the use of antibiotics. Several common candidates include penicillin's, cephalosporins, carbapenems and fluroquinolones. The choice of antibiotic is influenced by various factors such as the susceptibility of the identified pathogen, infection severity and potential for toxicity or adverse events (Bus et al., 2019).

Oral administration of these antibiotics is considered to be the primary treatment option due to the many benefits this route of administration provides; however, these treatments can also be delivered systemically in the case of moderate to severe infection. Topical administration of antibiotics such as vancomycin has been documented in the infection management of DFU, successfully promoting wound healing and reducing methicillin-resistant *Staphylococcus aureus* (MRSA) cultures in DFU when compared to saline dressing control (Saif et al., 2019). In instances where the patient is unresponsive to antibiotic treatment to manage DFU infection, surgery may be required.

For mild infection, NICE recommend the appropriate antibiotic to be administered orally for up to seven days (National Insititute for Health and Care Excellence (NICE), 2015). For moderate infection, primary choice of antibiotic should be administered, either orally or intravenously, for greater than seven days, or for six weeks in the case of osteomyelitis. For severe infection, combination treatment of antibiotics should be considered, as well as more frequent administration (National Insititute for Health and Care Excellence (NICE), 2015). Further insights into commonly used antibiotics will be discussed in a later in this review.

2.2. Advanced therapies

Often standard therapies such as those mentioned above are not themselves sufficient to allow complete DFU healing and prevent their reoccurrence. The failure of standard therapies to prevent the progression of mild DFU into moderate to severe DFU signals the requirement of advanced therapies to be initiated in the treatment strategy. Adjuvant therapies are provided with the aim of lowering the risk of re-infection and maximise the effectiveness of the wound healing process. Commonly used advanced therapies in the treatment of DFU are summarised in Table 2.

2.2.1. Advanced wound dressings

Following initial debridement and cleaning of the area, wound dressings provide moisture to the site of injury. Wound dressings produced from various materials exist and the choice of the most appropriate material for fabrication must be decided based on the type of wound, its severity and characteristics. The correct choice can promote healing of DFU as well as providing patient and economic benefits due to reduced treatment times. Films used in the treatment of DFU include hydrocolloids, alginates, foams, and silver-impregnated dressings, although hydrogels are used most commonly.

Hydrogels are “two- or multi-component systems consisting of a three-dimensional network of polymer chains and water that fills the space between macromolecules” (Ahmed, 2015). One study found hydrogels to be more effective when compared to basic wound dressings in the treatment of DFU, while non-adherent dressing provided more cost-effective benefits in comparison to hydrofiber dressings (Motley et al., 2015). Hydrogels can be laden with cells, such as mesenchymal stem cells (Chen et al., 2015), or growth factors, such as recombinant human epidermal growth factor (Hajimiri et al., 2016), and have proven promising in the promotion of DFU healing.

Hydrogels provide the benefit of tuneable properties, such as porosity, whilst also mimicking the natural granulation tissue which promotes biocompatibility (Chai et al., 2017). These hydrogels can however undergo large burst release phases, reducing their viability in the treatment of chronic ulceration where sustained drug release would be more effective.

The characteristics of the hydrogel largely depends on the chosen polymer. Synthetic polymers such as polyethylene glycol (PEG) are commonly used due to their desirable mechanical properties and, although they display good biocompatibility, natural polymers such as chitosan are generally more successful in closely mimicking biological structures such as ECM.

In a study hydrophilic polymers were used for the construction of hydrocolloids, where sodium alginate displayed the more desirable swelling properties, mechanical properties and greatest adherence to biological tissue (Jin et al., 2016). Vicenin-2-loaded sodium alginate hydrocolloids have been shown to reduce pro-inflammatory cytokines under hyperglycaemic conditions and promoted diabetic wound healing in an *in vivo* rat model (Tan et al., 2019).

2.2.2. Negative pressure wound therapy

In Negative Wound Pressure Therapy (NPWT), negative pressure is applied to a covered wound with the primary aim of removing fluids and infection to prepare a wound bed suitable for healing. NPWT promotes healing of acute and chronic wounds through use of negative pressure in a closed system (Muhammad et al., 2015).

NPWT has been found to decrease mRNA expression of pro-inflammatory factors such as IL-1B and MMP-9, while upregulating VEGF and TGF-B1 expression (Karam et al., 2018). In one study, IL-6 and TNF- α expression and inducible NOS production was suppressed significantly following seven days of NPWT treatment, but no significant difference was observed for the expression of p38 or extracellular signal regulated kinases 1 and 2 (T. Wang et al., 2019). NPWT significantly reduces oxygenation levels in diabetic feet and improves blood flow and micro-vessel maturation in diabetic wounds (Ma et al., 2017, (Jung et al., 2016). Furthermore, this treatment method has been noted to promote a moist environment around the wound, which is favourable for healing (Junker et al., 2013).

The non-invasive nature of this procedure makes it attractive for the use of DFU treatments. Many studies have been conducted to investigate the effectiveness of NPWT *versus* conventional DFU treatments such as wound dressings. Findings are however contradictory, with some studies have suggested NPWT is not more effective in the treatment of DFU compared to moist wound dressings (Seidel et al., 2020).

A recent meta-analysis by Liu et al., found NPWT to reduce the size of DFU and result in faster healing when compared to standard dressings (Liu et al., 2017). This analysis however also concluded the methodological flaws, such as lack of information, how DN affected DFU formation and progression, and the need for further investigation. NPWT provided significantly greater reduction in wound size and duration of hospital stay when compared to moist wound dressings; however, there was limited effect on the reducing the frequency of amputations resulting from DFU (Abdullah Al-Mallah, 2018).

NPWT treatment can be modified to improve the DFU healing profile. NPWT using Vacuum Assisted Closure (VAC) has been found to be more efficient in the reduction of wound area and more rapid healing times in long-term diabetic patients when compared to Advanced Moist Wound Therapy (AMWT) (Muhammad et al., 2015). A recent case study showed the promising benefits on wound healing when using a collagen contact layer, with 91% wound

healing occurring within 35 days (Lehrman, 2020), indicating the potential benefit of this material in future NWPT applications.

2.2.3. Skin substitutes

The increasing use of skin substitutes in the treatment of DFU is owed to their successful outcomes. Skin grafts such FDA-approved Dermagraft® have shown improved healing of chronic wounds, without adverse events (Marston et al., 2003). These skin substitutes mimic natural ECM, providing desirable biocompatibility, and provide a platform for the loading of cells and growth factors to promote wound healing. Despite desirable biocompatibility properties, use of skin grafts, as with any foreign material, poses risk for rejection from the body. This risk can be negated through various methods, including the use of bioengineered membranes composed of materials such as collagen. The potential of this method has been demonstrated through acellular membranes bioengineered with collagen, proteoglycans and glycoproteins, resulting in delayed onset of rejection *in vivo* (Stubenitsky et al., 2009).

Ananian et al., found viable cryopreserved placental membrane (vCPM) to have better wound healing outcomes and cost-effectiveness when compared to human fibroblast-derived dermal substitutes (hFDS) (Ananian et al., 2018). Additionally, a study showed that SIKVAV (Ser-Ile-Lys-Val-Ala-Val) peptide-modified chitosan hydrogel displayed more rapid wound healing, with greater collagen deposition, myofibroblast deposition and angiogenesis, compared to positive and negative controls (Chen et al., 2018).

3. Scaffold fabrication methods for DFU

It is well-established that traditional treatment options such as wound dressings, while biocompatible, lack desirable mechanical properties. An effort has therefore been made in the development of 3D scaffolds, which can be fabricated through several methods including electrospinning and 3D printing technologies.

To promote the cell adhesion and proliferation required for successful wound healing, highly porous scaffolds that closely mimic the ECM are required. These constructs must also have satisfying mechanical properties. Various techniques can be implemented to produce scaffolds of controlled porosity, mechanical strength and polymeric composition for example, depending on required application.

These scaffolds can be fabricated from a wide variety of biomaterials, which offer excellent biocompatibility profiles, while also providing structural support for the wound during tissue repair. In addition, composite scaffolds are becoming increasingly popular due to the dual benefits in terms of biocompatibility and mechanical properties offered from the biodegradable polymers used in their production.

This section will cover some of the most promising techniques used in the production of 3D scaffolds for DFU treatment. Table 3 summarises the advantages and disadvantages of each scaffold fabrication method.

3.1. Electrospinning

Electrospinning is a “process to obtain polymer, ceramic, metallic, and composite fibers from solutions, dispersions, or melts as a liquid jet accelerates through an electric field” (Laudenslager and Sigmund, 2012). The plastic syringe and syringe pump, shown in Figure 2, are used to expel the liquid jet. During the electrospinning process, an electric field is generated by the high power voltage supply and has a greater surface tension than that of the droplet. This produces a liquid jet, which is subjected to electrostatic repulsion within the Taylor cone and collected by the grounded collector. Solidification of the liquid jet occurs following solvent evaporation and results in a fibrous membrane (Qin, 2016). Figure 2 shows a schematic representation of the apparatus setup used for electrospinning.

One advantage of electrospinning is its ability to produce mats of high porosity, allowing them to mimic the ECM and making electrospinning an attractive option for the fabrication of scaffolds in the treatment of DFU. The porosity and pore size of the scaffold can be fine-tuned and adjusted in order to facilitate diffusion. Further advantages of the highly porous structures achieved by electrospinning includes the ability of these structures to act as physical barrier to microorganisms and allows oxygen to infiltrate the wound area to support healing (Samadian et al., 2020). Static, processing and environmental parameters can be optimised to ensure reproducibility of the electrospinning process, while parameters such as polymer concentration and voltage can be modified to achieve the desired scaffold architecture and characteristics.

The resulting high surface area to volume ratio of these porous scaffolds provides opportunity for higher drug loading at site-specific delivery, which would be particularly beneficial for sustained drug delivery. Moreover, the porous structure of the scaffold would aid in maintaining a moist wound area through fluid accumulation, which is highly desirable for

effective wound healing (Junker et al., 2013). Moreover, a broad selection of both natural and synthetic polymers has been used in the construction of electrospun scaffolds for DFU healing. Despite this method being easy to scale-up, additional processing steps to allow for crosslinking may be required, which has the disadvantages of extending production time and extra costs.

Overall, electrospinning allows the production of scaffold fibers to the nanoscale, providing benefits such as increased surface area for cellular adhesion.

Both uniaxial and coaxial electrospinning methods have been employed to fabricate scaffolds for wound healing applications. Ren et al., demonstrated the role of highly aligned fibres using uniaxial electrospinning in significantly enhancing cellular attachment during wound healing (Ren et al., 2018). This process however required highly volatile solvent to achieve the highly porous structure. These nanofiber constructs have proven successful in localised delivery of wound healing agents, which results in complete and successful wound healing of DFU.

Progressing from traditional simple nanofiber constructs, there has been greater development of composite nanofiber scaffolds. Ahmed et al., found significant contraction of the wound area in an *in vivo* diabetic rabbit model for the chitosan/polyvinyl alcohol/ZnO scaffolds when compared to chitosan/polyvinyl alcohol scaffolds, as well as higher antibacterial and antioxidant potential (Ahmed et al., 2018). Curcumin-loaded scaffolds, which were generated from gum tragacanth/PCL electrospun fibres showed significant wound closure following 15 days and displayed functions characteristic of normal wound healing, such as increased collagen deposition, well-formed granulation tissue and complete regeneration of the epithelial layer. The electrospinning method allowed well-distributed cellular attachment to these scaffolds (Mohammadi et al., 2016). Core-shell scaffolds produced from chitosan and PLA through the coaxial electrospinning technique demonstrated feasibility in its application to tissue engineering, having increased elastic modulus and tensile strength of the scaffold as well as supporting cell adhesion and proliferation of fibroblasts (Surucu and Turkoglu Sasmazel, 2016).

Electrospinning has also been used in combination with 3D printing methods to produce a scaffold which successfully mimicked the ECM, promoted cell proliferation and diabetic wound healing in an *ex vivo* pig model (Mellor et al., 2017).

The relative advantages and disadvantages of electrospinning scaffold fabrication methods are summarised in Table 3.

3.2. 3D Printing: Fused Deposition Modelling

Fused deposition modelling (FDM) is a “technology where the melt extrusion method is used to deposit filaments of thermal plastics according to a specific pattern” (Walker and Santoro, 2017). Prior to printing *via* FDM methods, the user produces a scaffold design using Computer Aided Design (CAD) which is then subject to slicing to allow printing. Using this technology, filaments of thermal plastics are extruded through a nozzle using the melt extrusion method in a predetermined pattern, which corresponds to the chosen CAD design. This process follows a layer-by-layer approach to fabricate scaffolds of various geometries and sizes. Figure 3 shows a schematic representation of the apparatus setup used for FDM.

The architecture of the porous scaffolds used in these wound healing applications can be easily designed and altered using CAD software. Like electrospinning, this method can produce scaffolds composed of a wide variety of polymeric materials, based on the desired properties of the scaffold. Scalability and rapid production of scaffolds, in a timescale of minutes to hours, provide further advantages for this method. Scaffolds produced by FDM have shown promising mechanical properties, no cytotoxicity and acute enhancement of fibroblast proliferation (Kovalcik et al., 2020).

Chitosan-based scaffolds produced by FDM showed significant cellular proliferation and infiltration by keratinocyte and fibroblasts *in vitro*, with optimal cell growth achieved following 35 days. These structures lead to accelerated healing of the diabetic wound *in vivo* and successfully prevented wound infection (Intini et al., 2018). FDM printing was used to produce a scaffold, which had an elastic modulus equal to that of collagen (24MPa) which provided optimal conditions for normal wound healing, such as polarisation of macrophages towards the M2 phenotype and increased expression of the PDGFb gene in granulation tissue (Guo et al., 2015).

FDM does not require the use of solvents, as successful extrusion of the material can be achieved through optimisation of temperature, and pressure parameters and has suggested to be more useful for production of larger scaffolds (Trachtenberg et al., 2016). As well as this, FDM is capable of printing high viscosity solutions while maintaining control over aspects such as pore size and degree of porosity (Trachtenberg et al., 2016)

Meshes fabricated through 3D printing have shown to improve mechanical properties of electrospun PCL/gelatin scaffold *in vivo* whilst maintaining a structure which closely mimicked ECM (Pensa et al., 2019). FDM is capable of producing scaffolds with a higher Young's modulus *versus* solvent exchange deposition modelling (SEDM) (Gao et al., 2020). Due to the nature of the skin however, these structures produced by FDM may be too rigid and not offer the flexibility required for these applications. In this case, the combination of FDM with other scaffold production techniques such as SEDM or electrospinning should be considered. Furthermore, subject to the material used, combination of high shear, high temperature and high pressures required for this process may subject the material to phase changes and alterations, which ultimately affect the biocompatibility of the given material (Domingos et al., 2009).

FDM printing has been used in combination with other processing techniques. Song et al., achieved microporous PLA scaffolds through the combination of FDM and gas foaming processing for bone tissue engineering purposes. The FDM process produced macropores of 100 – 800µm in size, while micropores of 2 – 10µm were also produced due to gas foaming (Song et al., 2018). This work shows the potential of technologies such as gas foaming to be used in a complimentary manner with FDM to improve the resolution of FDM printing which could be applied to diabetic wound healing applications.

The relative advantages and disadvantages of FDM scaffold fabrication methods are summarised in Table 4.

3.3. 3D Bioprinting

In the production of 3D bioprinted scaffolds, the bioink is laid down in a layer-by-layer manner to fabricate a pre-specified design, allowing the user to create scaffolds with a high degree of repeatability, which mimic the target organ or tissue. Similar to FDM, in the pre-processing stage scaffolds designs are created using CAD and are subject to slicing in preparation for bioprinting. 3D bioprinting techniques, collectively known as additive manufacturing (AM), includes inkjet-based, extrusion-based and laser-based technologies. The chosen bioprinting technique should take into account factors such as the application of the scaffold, the materials used and the corresponding benefits and drawbacks of each bioprinting method.

In general, bioprinting offers several advantages, including the production of scaffolds with a highly porous structure that are capable of supporting cell adhesion and proliferation and

allowing oxygen to permeate through the structure to ensure nutrient transport to the innermost sections of the scaffold. High loading of cells and growth factors can also be achieved (Wang et al., 2021), although the extent to which this is achieved varies between bioprinting methods. For example, stem cells, namely adipose-derived stem cells (ASCs) and endothelial progenitor cells (EPCs) have been incorporated into a skin-derived ECM bioink for wound healing applications. This patch was found to successfully enhance wound closure, improve blood flow in the early wound healing stages and promote re-epithelialisation (Kim et al., 2018).

The relative advantages and disadvantages of various bioprinting techniques are summarised in Table 5.

3.3.1. Inkjet-based bioprinting

Inkjet-based bioprinting is a low-cost method involving droplets of solution being dispensed in a non-contact manner to create a pre-specified design. This can be done using thermal, piezoelectric or micro-valve mechanisms (Rider et al., 2018). The size and volume of these droplets can be carefully controlled, ultimately producing high-resolution scaffolds, as well as highly complex structures when required. Figure 4 shows the schematic setup of ink-jet based bioprinting technologies.

The rapid inkjet-based bioprinting process involves two stages: firstly, the deposition of bioink droplets on a picolitre scale onto the substrate secondly followed by *in situ* gelation of the biomaterial to form a solid structure. In some cases, chemical, physical, or photo-crosslinking of the chosen biomaterial may be required in order to maintain the structural rigidity of the scaffold. A rapid gelation time is therefore required in order to prevent spreading of the printed bioink which would lead to poor shape fidelity of the resulting structure.

Despite rapid printing speeds, inkjet bioprinting cannot achieve high cell loading densities (Negro et al., 2018). Still, a high cell viability can be achieved, with some studies achieving up to 99% viability (Takagi et al., 2019). Furthermore, high resolutions of 100µm have been reported using inkjet bioprinting methods (Zhang et al., 2017).

Two forms of inkjet-based bioprinting exists: continuous inkjet (CIJ) printing and drop-on-demand (DOD) printing. In CIJ, a piezoelectric crystal attached to the printing nozzle sends ultrasonic vibrations to break up the continuous ink Jetstream into small droplets. Some of these droplets become charged as they pass through an electrostatic field before passing

through a second electrostatic field, causing charged droplets to deflect towards the printing medium (Needham Coding, 2020). Polymer degradation has been observed as a result of bioink recycling during CIJ (Wheeler et al., 2014). This could have significant implication for scale up activities, including loss of a large amount of material and therefore this method should be carefully considered for large-scale activities. Meanwhile, DOD achieves higher precision and accuracy through use of a mechanism which only produces droplets when an ejection signal is reached (Haas et al., 2017).

Inkjet bioprinting has been used for direct printing of cells and biologics such as growth factors. Yanez et al., used inkjet bioprinting method to produce a bilayer skin graft in where one collagen-based layer was laden with neonatal human dermal fibroblasts and epidermal keratinocytes, while the fibrin layer was laden with endothelial cells. Overall, this scaffold demonstrated 17% improvement in wound contraction compared to FDA-approved Apligraf (Yanez et al., 2015)

Inkjet bioprinting devices capable of *in situ* bioprinting have recently been developed in an effort to overcome typical problems such as irregular wound topology (Albanna et al., 2019).

3.3.2. Extrusion-based bioprinting

In extrusion-based bioprinting, the ink is extruded through the nozzle in a continuous manner, which is driven by either a piston, screw or pneumatic pressure mechanisms, allowing the user to fabricate a product in a layer-by-layer manner. This affordable method is preferred for the preparation of larger structures and has been identified as the most suitable bioprinting method for soft tissue purposes (McCormack et al., 2020). Parameters such as nozzle diameter, pressure, temperature and extrusion speed can be altered depending on the material used and its application. Figure 5 provides an outline of the setup for extrusion-based bioprinting technologies.

Although rare in literature, given studies have demonstrated the ability of extrusion-based bioprinting to successfully extrude viable cells using nozzle diameters of 200µm (Kolesky et al., 2016). Typically, compared to inkjet bioprinting, more moderate resolution values have been recorded in the range of 300µm to 600µm (Tan et al., 2016). Parameters affecting resolution include printing conditions (e.g. pressure), material properties, material extrudability and print time (Gillispie et al., 2020). Unlike inkjet-based bioprinting however, extrusion-based bioprinting allows high seeding capacities (Tan et al., 2016).

Screw-driven extrusion is suitable for high viscosity materials such as PCL (Visser et al., 2013), primarily due to the level of control it provides over the flow of the bioink. Higher viscosity bioinks result in greater resolution (Zhuang et al., 2019). Despite this, printing at a high pressure will incur issues such as the requirement of a faster printing speed which will in turn reduce cell viability (Fakhruddin et al., 2018)

Various cell lines have been investigated in association with extrusion-based bioprinting. Each cell type will have different viability values dependent on their given ability to withstand shear stresses involved with the printing process.

Soltan et al., demonstrated the use of extrusion-based bioprinting to produce alginate dialdehyde-gelatin hydrogels for tissue engineering purposes. It was found the printability of scaffolds bioprinted at both room temperature and 4°C could be improved with the presence of a chemical crosslinker. In addition, cell viability of HUVECs and Schwann cells was maintained for greater than 7 days (Soltan et al., 2019). In order to promote tissue repair, good cell viability and printability of materials is essential, thus demonstrating the potential this scaffold has for wound repair. Cubo et al., used extrusion-based bioprinting to produce a bilayer scaffold to closely mimic the skin. Laden with human plasma, keratinocytes and fibroblasts, these scaffolds demonstrated the suitability of extrusion-based bioprinting to produce structures which closely mimic the skin (Cubo et al., 2016).

Extrusion-based bioprinting techniques are capable of producing scaffolds with desirable mechanical properties. Frost et al., incorporated cellulose nanocrystals (CNC) into PEGDA scaffolds, resulting in scaffolds with excellent mechanical properties, which could easily be tuned by varying CNC concentration. As a further advantage, these complex scaffolds could be fabricated at low temperatures and pressures (Frost et al., 2019).

3.3.3. Laser-assisted bioprinting

In this bioprinting technique, an infrared-pulsed energy source is focussed on a ribbon coated with liquid biological materials, which is spread on a metal plate. Radiation then causes removal of the ribbon through evaporation of the biological materials. These reach the substrate in droplet form (Sriphutkiet et al., 2019, Li et al., 2016). Figure 6 outlines the setup of laser-assisted bioprinting technologies.

Laser-based bioprinting allows printing of biological material, such as cells and DNA at high speed printing of cells, in a MHz range, at a high resolution (Guillotin et al., 2010). High cell densities can also be achieved. Kerouredan et al., showed application of laser-assisted bioprinting for tissue engineering purposes, demonstrating the ability of this technique to allow controlled printing of endothelial cells co-cultured with MSC at high densities within a collagen hydrogel for the formation of vascular structures (Kérourédan et al., 2019). Laser-assisted bioprinting offers resolution values superior to that of both inkjet-based and extrusion-based bioprinting and depends on parameters including but not limited to the layer thickness, surface tension and bioink viscosity.

Laser-assisted bioprinting technique does not require a nozzle, therefore eliminating issues such as nozzle clogging, which are observed with inkjet-based and extrusion-based bioprinting methods (Li et al., 2018), and allows for greater reproducibility. Furthermore, the non-contact nature of this bioprinting method conveys a promising strategy for *in situ* bioprinting. The application of laser-assisted bioprinting has been demonstrated for bone engineering purposes, showing the ability of this technique to allow controlled *in situ* printing of endothelial cells, which resulted in bone regeneration (Kérourédan et al., 2019).

Drawbacks of this method include the time-consuming preparation of the ribbon, particularly if several cell lines are required, and the expensive nature of the technique. The laser source has been noted as a potential disruption to cell viability (Guillotin et al., 2010); however, studies using laser-assisted bioprinting have demonstrated high cell viability (Keriquel et al., 2017), indicating this to be a minor occurrence/uncommon issue.

4. Suitable materials for 3D bioprinted scaffolds

Wide varieties of polymeric materials are currently being used for 3D bioprinting. The suitability of the material to a particular application comes down to its printability, which in turn is influenced by various properties such as the ability of the material to undergo rapid gelation following extrusion. Importantly, the material should impart certain characteristics that allow the final scaffold to possess similar mechanical, rheological and biological properties of the therapeutic target (Gungor-Ozkerim et al., 2018).

Polymers have been reported in the literature extensively for the fabrication of 3D bioprinted scaffolds for the treatment of DFU. Polymers can be classified as either biodegradable or non-biodegradable, but in the case of 3D bioprinting, biodegradable polymers are used more

frequently due to the wide variety of benefits they possess. Depending on their origin, these biodegradable polymers are natural or synthetic and have been used extensively in wound healing applications for DFU, both alone and in combination.

4.1. Natural polymers

Natural polymers have shown excellent biocompatibility profiles, which is essential in preventing a foreign body response from the host. Natural polymers however sometimes lack the desired mechanical properties that the scaffolds require in order to provide structural support to the wound healing process. Nonetheless, the popularity of these materials remains high. Natural polymers commonly used in 3D bioprinting are summarised in Table 7.

4.1.1. Collagen

As the main structural protein in ECM, collagen is a commonly used material in 3D bioprinting applications for the skin, primarily due to how closely it mimics the physiochemical properties of the skin. A non-toxic and highly permeable material, collagen provides many advantages for wound healing applications and provides the greatest biocompatibility profile of natural polymers (Skardal and Atala, 2015). pH neutralisation at a temperature of 37°C is required for gelation of collagen (Carolina et al., 2020).

Common issues associated with collagen in wound healing applications include its poor mechanical properties and low viscosity (Dong and Lv, 2016). In order to overcome this issue, incorporation of a synthetic polymer may lead to improved mechanical properties whilst preserving the excellent biocompatibility properties that collagen imparts to the bioprinted scaffold. The addition of elastin-like peptide saw a threefold increase in the modulus of collagen scaffold (Gurumurthy et al., 2016). A further way of improving the mechanical issues associated with collagen is through crosslinking (Bou-Akl et al., 2013). This can be achieved through chemical or physical crosslinking methods. In literature, common chemical crosslinking methods for collagen matrices include, but are not limited to, the use of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC)/N-hydroxy-succinimide (NHS) (Davidenko et al., 2015), genipin (Zhou et al., 2018) and glutaraldehyde (Tian et al., 2016). Physical crosslinking methods have witnessed the use of dehydrothermal treatment (Kozłowska et al., 2017) and UV (Heo et al., 2016).

Collagen forms a hydrogel under physiological conditions; however longer timeframes of 30 minutes and above are required for this process to occur. To reduce these times, and thus improve printing accuracy, the combination of other materials should be considered (Gopinathan and Noh, 2018)

4.1.2. Gelatin

Gelatin, is another commonly used biodegradable polymer for biomedical applications, which is formed through denaturation of collagen's triple helix (Boccafroschi et al., 2019). The wide availability, low cost, low immunogenicity, high solubility and structural similarities to collagen make gelatin a popular biomaterial (Bello et al., 2020).

Due to issues experienced as a result of reversible temperature-dependent sol-gel transition of gelatin, native gelatin is rarely used alone in tissue engineering applications (Cheng et al., 2019). As a result, to overcome printability issues, gelatin is commonly used alongside other suitable biomaterials in the fabrication of 3D bioprinted scaffolds. A lack of thermal stability and faster degradation rate of gelatin are among some of the materials disadvantages, as well as the typical poor mechanical properties witnessed with other natural polymers (Bello et al., 2020).

Gelatin-alginate composites have been used extensively in literature for wound healing applications. Liu et al., demonstrated the ability of gelatin-alginate scaffolds in reducing the wound healing time in an *in vivo* diabetic rat model. The scaffold provided benefits such as reduced necrosis, even granulation tissue formation and reduced swelling (Liu et al., 2016). Moreover, gelatin-alginate bioink combinations have demonstrated the ability to promote cell proliferation and differentiation, good injectability, stability towards cells, regular pore structure and mechanical strength similar to that of the mouse dermal tissue (Cheng et al., 2019)

Modified forms of gelatin, such as gelatin methacryloyl (GelMA), have also been studied in literature. In wound healing applications, GelMA improved angiogenesis and enhanced proliferation fibroblasts and keratinocytes in the form of electrospun patches to promote healing of diabetic wounds *via* release of epidermal growth factor (EGF) (Augustine et al., 2021).

4.1.3. Alginate

As a naturally derived anionic polymer from brown algae, alginate has proven promising in wound healing applications. This can be attributed to its desirable properties including biocompatibility, non-toxicity and low cost.

Alginate is a linear copolymer composed of alternate blocks of (1,4)-linked β -D-mannuronate (M) and α -L-guluronate (G) residues (Gaspar et al., 2016). The addition of divalent cations such as Ca^{2+} for crosslinking purposes is known to lead to the gelation of alginate, and the resultant formation of a hydrogel suitable for biomedical application (Gwon et al., 2015). Only guluronate blocks contribute to crosslinking with divalent cations in the formation of hydrogels, meaning the composition of copolymer blocks within the alginate structure is essential to determining its physiochemical properties and feasibility as a 3D bioprinted scaffold (Chuang et al., 2017).

As a biomaterial, alginate can easily be modified. Thomas et al., retained the beneficial properties of alginate whilst undergoing modification through addition of aloe-vera gel and CNC to further improve antibacterial and mechanical properties in a conventional wound dressing application (Thomas et al., 2020). Its other advantages include the ability to maintain a moist microenvironment to support wound healing and prevent scar formation (Hana et al., 2014).

As discussed above, alginate has commonly been used in combination with gelatin in order to enhance the mechanical properties of the two biomaterials. In addition to this, the inclusion of CaCl_2 as a chemical crosslinking agent has been shown as a successful method in improving both the mechanical and physical properties of sodium alginate films, with 0.8M of CaCl_2 for 8 minutes producing a Young's modulus of 27.81 ± 7 MPa, as shown in Figure 7 and Table 7 (Fadhilah et al., 2019).

Alginate forms a gel in the presence of CaCl_2 , between 5mM/L and 6.25 mM/L of CaCl_2 for every 2%w/w alginate solution (H. Li et al., 2016). However, at a concentration of 2%w/w, alginate cannot form scaffolds due to poor mechanical properties. Increasing the concentration of alginate and the addition of graphene oxide have been reported to limit the effects of the materials shear-thinning behaviour, making it more suitable for bioprinting (H. Li et al., 2016). Given the poor mechanical properties of alginate, it is commonly used in combination with other biomaterials.

4.1.4. Fibrin

A potent haemostatic agent, fibrin acts to promote cell adhesion and has desirable biocompatibility in wound healing applications (Climov et al., 2016; Robinson et al., 2017). As a bioink however, fibrin presents several issues.

The rapid degradation of fibrin remains a major obstacle in its use for 3D bioprinted scaffolds; however, recently this issue has been addressed in literature through addition of chemical crosslinking agents, such as genipin (Robinson et al., 2017). Losi et al., developed poly(ether)urethane–polydimethylsiloxane/fibrin-based scaffold for the delivery of nanoencapsulated VEGF and bFGF. These scaffolds were found to significantly increase wound healing capabilities in an *in vivo* diabetic mouse model, with wound closure occurring at day 15 (Losi et al., 2013). Fibrin also requires a long gelation time of 30 minutes (Cubo et al., 2016)

As with many natural polymers, scaffolds fabricated from fibrin possess poor mechanical properties. In addition to this, proper extrusion of fibrin can be compromised due to the high viscosity of the biomaterial. Similarly, poor shape fidelity is witnessed when printing fibrinogen (de Melo et al., 2020). One strategy used to overcome this issue involves using fibrinogen in combination with other biomaterials, such as gelatin and polyethylene glycol, which show desirable printing properties.

4.2. Synthetic polymers

Prepared by chemical synthesis, synthetic polymers used in 3D printing provide benefits such as improved mechanical properties, which helps to aid the properties of natural polymers when used in composite scaffolds. Further general advantages of synthetic polymers include the ability to easily tune their degradation rates, easy modification and functionalisation of their structures and their accessibility. Synthetic polymers commonly used in 3D bioprinting are summarised in Table 8.

4.2.1. Poly(lactic-co-glycolic acid)

Poly(lactic-co-glycolic acid) (PLGA) is a copolymer consisting of lactide and glycolide monomers and is synthesised through ring opening of these cyclic dimer rings (Teodora et al., 2015). As well as being biocompatible, PLGA provides desirable mechanical properties for

scaffold applications, is a low-cost material and is highly accessible. The degradation rate of PLGA can be easily tailored through manipulation of the monomer ratio, with higher percentage of glycolic units resulting in extended degradation rates (Keles et al., 2015)

PLGA is one of the most commonly used materials in biomedical applications, including hydrogels, nanoparticles and films. PLGA has been used in combination with natural polymers in wound healing scaffold applications. A bilayer scaffold composed of PLGA and alginate, as shown in Figure 8, showed promising wound healing abilities, with PLGA playing a particular role in maintaining moisture content of the hydrogel and inhibiting infection (Wang et al., 2019).

One study concluded that in order to achieve high fidelity through printing of PLGA, concentrations greater than 80%w/v are required, as well as a higher ratio of lactic acid. Furthermore, a molecular weight greater than 100kDa and internal nozzle diameter less than 0.96mm is also recommended (Naseri et al., 2020).

One drawback of PLGA in 3D printing applications is that high temperatures and pressures are required during printing (Allevi, 2020).

4.2.2. Polycaprolactone

Polycaprolactone (PCL) is a high molecular weight semi-crystalline thermoplastic polymer, which demonstrates desirable biocompatibility characteristics. Its low melting point of 60°C and gelation temperature of 37°C makes it appealing for 3D bioprinting due to its ease of processing (Burns et al., 2010, Nair et al., 2017). PCL undergoes a two-stage degradation process involving hydrolysis and intracellular degradation, with a degradation time of two to four years (Azimi et al., 2014). Moreover, PCL and its degradation products are non-toxic (Sukanya and Mohanan, 2018).

One drawback of PCL in bioprinting is the higher pressures required due to the high viscosity of the material (Cellink, 2020). Support of cellular attachment from scaffolds is an integral part in their work to aid wound healing of DFU; however, the hydrophobic nature of PCL can mean cell colonisation is insufficient. To overcome this, various strategies can be applied to improve the hydrophilicity of PCL. Techniques applied include chemical methods involving the addition of NaOH, for example (Wang et al., 2016). Higher viscosity can also increase the printing time.

Despite this, PCL has been used extensively in 3D bioprinting applications for DFU wound healing. One study investigating the effect of various nozzle shapes and sizes, printing speeds and printing temperatures concluded large nozzle sizes and high printing speeds produced the optimal printing conditions when compared to cylinder nozzles (Ortega et al., 2019).

4.2.3. Polyurethane

Polyurethane (PU) is a biocompatible material prepared *via* the addition reaction of diisocyanate and a polyol, followed by the addition of a chain extender, to create a polymer chain comprised of various hard and soft segments (Balaji et al., 2018). The chemical composition of PU directly affects key properties such as thermosensitivity and biodegradability (Tsai et al., 2015).

The excellent mechanical properties and ease of processing of PU makes it an attractive option for scaffold bioprinting applications. Furthermore, as well as its shear thinning properties, PU also shows good elasticity and biocompatibility (Hsieh and Hsu, 2019). As a thermosetting material, scaffolds fabricated from PU demonstrate thermoplastic and elastomer properties.

The biodegradability of PU can be improved through the addition of various other polymers. When used in combination with gelatin, the resultant hydrogel showed excellent printability at low temperatures (24 – 31°C), high fidelity, shear-thinning, recovered quickly after excessive strain, could be bioprinted for more than 24 hours, could be stacked well during printing and showed good cell viability and proliferation over a ten-day period. Furthermore, chemical crosslinking with CaCl₂ improved mechanical properties such as the compression modulus (Hsieh and Hsu, 2019).

4.2.4. Polyethylene glycol

Polyethylene glycol (PEG) is a biocompatible, hydrophilic polymer that is naturally non-biodegradable, however modification of the PEG structure to include degradable segments induce biodegradable properties.

Composite scaffolds composed of sodium alginate and PEG loaded with *Satureja cuneifolia* have shown promise in wound healing applications due to suitable swelling behaviour, pore size and antimicrobial activity (Ilhan et al., 2020). GelMA/gelatin/PEG bioprinted scaffolds modified with silica nanoparticles promoted macrophage polarisation towards the anti-

inflammatory M2 phenotype in diabetic bone repair in an *in vivo* rat model. As polarisation of macrophages towards M2 phenotype is compromised in diabetic wound healing, this approach could be applied to 3D bioprinted scaffolds for diabetic wound healing applications.

Cellular adhesion cannot be achieved for PEG hydrogels, therefore modification of PEG through the addition of acrylate groups to form poly(ethylene glycol) diacrylate (PEGDA) provides a medium for the adhesion and support of cells (Asawa et al., 2018). The addition of PEGDA as a hydrophilic polymer helped to improve the hydrophilicity of composite scaffolds fabricated from PCL/PEG/chitosan (Cheng and Chen, 2017).

5. Common drugs used in the treatment of DFU

As mentioned previously in Section 2, the treatment of DFU may involve the use of standard therapies in combination with advanced therapies. The overall aim of treatment however is to prevent or eradicate infection, encourage a normal wound healing response and control factors such as hyperglycaemia, PAD or DN which could negatively impact the wound healing process. To meet each of these aims, a combination of drugs is often required. In this section, we will review some of the commonly used drugs used in the treatment of DFU.

5.1. Antibiotics

One of the first steps to ensure successful wound healing of DFU is to treat any existing infection. Failure to treat any infected ulcers could result in colonisation of pathogens to form a biofilm, which often tend to be resistant to antibiotics and allow disease progression. Drug resistance and recurring infection has been associated with the formation of biofilms (Vatan et al., 2018).

The choice of antibiotic used is dependent on factors such as the severity of the ulcer and pathogen susceptibility, for example. IWGDF have published guidance on the treatment of infected DFU (Bus et al., 2019). Several commonly used antibiotics include penicillins, cephalosporins, carbapenems and fluoroquinolones.

In a long-term study conducted between 2008 and 2015, *Escherichia coli* and *Staphylococcus aureus* were the two most common bacteria found to infect diabetic wounds (Zubair and Ahmad, 2019). This is supported by more recent findings in where biofilms of *S. aureus* have been found to be promoted by AGEs, which are increased in diabetic patients (Xie et al., 2020).

May studies into the use of antibiotics to treat DFU infection involve small sample sizes and lack patients suffering from PAD and DN. Furthermore, most studies do not acknowledge the typical polymicrobial nature of DFU infections and biofilms. While many promising and effective treatment outcomes have been noted in literature, there is a strong need for larger studies, studies that focus of treating DFU in patients also suffering from PAD and DN and *more* studies that investigate polymicrobial biofilm nature of DFU. These aspects need to be addressed in order to achieve reliable data on infection management of DFU, which would be generalizable to clinical settings.

Nonetheless, there is growing evidence for the improved efficacy of topically administered antibiotics to treat biofilms in infected DFU versus systemic administration (Price et al., 2020). A small-scale study was conducted in 23 patients to compare the efficacy of a saline dressing loaded with 500mg vancomycin *versus* a non-drug loaded saline dressing against DFU infected with MRSA. The infection was resolved in approximately two thirds of patients within two weeks of topical vancomycin treatment, demonstrating its efficiency in the treatment of DFU infection in the absence of any systemic toxicity (Saif et al., 2019). While promising, larger sample sizes are needed.

Controversially, one study found the use of a collagen sponge loaded with gentamycin to have no significant benefit in the treatment of mild DFU infection, compared to the control group who did not receive treatment (Uçkay et al., 2018). Similar findings were obtained for moderate to severe DFU infections (Uçkay et al., 2018).

As well as conventional antibiotics, such as those mentioned above, broad-spectrum silver sulfadiazine has shown promising activity when applied topically to DFU. Low concentrations of silver sulfadiazine successfully eliminated DFU infection within 30 days in patients infected with *S. aureus* and *Pseudomonas aeruginosa* biofilms. (Di Domenico et al., 2020). The MBEC values for *S. aureus* and *P. aeruginosa* ranged from 1.25 to 2.5 mg/L and 0.16 to 0.31 mg/L, respectively. The reduction in inflammation and collagen deposition following two weeks of treatment indicate the key role infection management plays in the successful wound healing of DFU.

5.2. Human growth factors

Human growth factors such as PDGF, VEGF and EGF play a key role in orchestrating a normal wound healing process. For example, PDGF is responsible for promoting chemotaxis and

synthesis of fibroblasts and macrophages (Pierce et al., 1991), two key cells in the wound healing process. Significant association has been found between polymorphism of the VEGF gene and DFU (Talib et al., 2020). In addition, careful consideration is required for the use of TGF, as a balance must be achieved between iNOS and TGF-B1 (El-aleem et al., 2020).

Coaxial PLGA scaffolds prepared by electrospinning were loaded with PDGF, as well as antibiotics gentamicin and vancomycin, which resulted in increased angiogenesis leading to accelerated epidermal proliferation and wound healing in a diabetic rat model *in vivo* (Lee et al., 2020). When encapsulated in PLGA nanospheres, both PDGF and VEGF-a significantly reduced the ulcer size and promoted wound healing in a diabetic rat model (Shi et al., 2018). This method of delivery could overcome common issues associated with growth factors, such as susceptibility to degradation by enzymes.

In literature, EGF has shown effective in improving DFU healing rates. Complex coacervates fabricated from sodium alginate and gelatin were used to provide sustained release of EGF to diabetic mice *in vivo*. This system successfully lowered pro-inflammatory cytokines such as IL-1 by day 7 and achieved complete wound closure by day 14 (Jeong et al., 2020). Patches containing EGF have shown more favourable cell adhesion and proliferation by key cells, such as fibroblasts and endothelial cells, versus PHBV-GelMA patches, indicating the crucial and beneficial role EGF in improving the wound healing capabilities. Furthermore, the presence of EGF, and its possible positive influence on VEGF, promoted angiogenesis (Augustine et al., 2021). As angiogenesis is often insufficient in diabetic wounds, the presence of growth factor was paramount in achieving a positive healing response of the diabetic wound.

Kontopodis et al., investigated the effect of autologous platelet-rich plasma on diabetic wound healing. Nearly three quarters of patients with DFU and PAD experienced greater than 90% reduction in ulcer size and the lower limb amputation rate was considerably lower than the typical value quoted in literature (19% versus a reported 85%) (Kontopodis et al., 2016). This indicates platelet-rich plasma to be a promising therapy for diabetic patients simultaneously suffering from PAD; however, larger sample sizes are required to make this generalizable.

5.3. Stem cells

The use of stem cells to enhance DFU wound healing is another area receiving considerable attention. There is particular interest in mesenchymal stem cells (MSCs).

MSCs, which are found in bone marrow and adipose tissue, have proven useful in wound healing applications. Recently, adipose-derived mesenchymal stem cells preconditioned by photobiomodulation, firstly in vitro and then followed by in vivo, were found to significantly improve healing rates of ischemic infected DFU in a rat model (Ahmadi et al., 2020). MSCs provide a great number of benefits, such as their ability to manage oxidative stress and promote an anti-inflammatory response and formation of new blood vessels in damaged tissues (Becerra-bayona et al., 2020).

Case studies indicate the promising ability of allogenic bone marrow MSC derivatives administered *via* intradermal injection on healing grade 2 DFU, as classified by IWGDF, when compared to a combination treatment of allogenic bone marrow MBC and a conventional dressing. No difference however was observed in the derivative treatment or MSC treatment (Becerra-bayona et al., 2020).

The ability of human umbilical cord MSC to migrate to the site of DFU following intravenous administrated was demonstrated in a rat model. Treatment with human umbilical cord MSC lead to a gradual decrease in ulcer size over time, reduced inflammation, increased secretion of key growth factors such as VEGF, enhanced angiogenesis, promoted re-epithelialisation and reduced apoptosis (Shi et al., 2020), demonstrating the feasibility of IV administered huc-MSC to successfully treat DFU.

While studies in literature indicate promising results of stem cells for wound healing, many of these studies only examine the short-term efficacy of this treatment method. Longer studies are required in order to establish long-term effectiveness and safety of stem cells for wound healing applications.

5.4. Topical agents

While many topical agents have been investigated in the treatment of DFU, one group is receiving particular interest.

Monoterpenes, comprising of two isoprene units and 10 carbon atoms, belong to the class of drugs known as terpenes, or isoprenoids. Terpenes are the main constituents found in essential oils and demonstrate excellent wound healing capabilities due to their anti-inflammatory and antimicrobial properties (Buckle, 2015). Monoterpenes commonly used in wound healing applications include: borneol, thymol, α -terpineol, genipin, aucubin, d-Limonene and sericin.

Thymol when incorporated into a collagen-based film showed significant improvement in wound retraction on day 7 and 14 compared to wounds dressed with non-drug-loaded collagen films and the untreated control group. On day 7, thymol-collagen dressing groups showed formation of mature granulation tissue, well-formed blood vessels and denser deposition of collagen when compared to undressed and collagen dressed wounds (Riella et al., 2012).

An electrospun PCL patch, loaded with thymol (PCL-THY), which showed similar activity to corticosteroid dexamethasone was demonstrated, with an effective reduction inflammation when used in the PCL scaffold versus when used in combination with tyrosol or tyrosol alone. PCL-THY showcased a 7.33% burst release of THY in the first 60 min while PCL-TYR resulted in release of 61.74% after 8 h (García-Salinas et al., 2020). While promising wound healing capabilities were demonstrated, the drug release of this formulation should be modified in order to achieve sustained drug release over a period of weeks.

Other existing drugs are also showing promising results. Topical application of 0.5% timolol maleate, a beta blocker, in combination with antibiotics and dressings to diabetic ulcers found significant improvement in wound healing versus the control group who received only antibiotics and dressings, with an approximate 60% reduction in ulcer size by week 12 irrespective of ulcer type (Thomas et al., 2017). Similarly, successful treatment of DFU with the use of another beta blocker has been noted. Re-epithelialization, increased angiogenesis and ECM formation was observed in diabetic mice receiving 1% propranolol cream to chronic diabetic wounds (Zheng et al., 2017)

Previously, topical delivery of phenytoin has demonstrated effectiveness in the treatment of grade 1 and 2 DFU (Patil et al., 2013). Phenytoin is a commonly prescribed anticonvulsant used to treat seizures (National Institute for Health and Care Excellence, 2020), but is showing promising use as a wound healing agent. More recently, delivery of topical phenytoin through a nanostructured lipid carrier dressing demonstrated greater healing abilities of neuropathic DFU when compared to phenytoin hydrogel of the same concentration (Motawea et al., 2019).

Patients with Wagner stage 1 and 2 DFU treated with daily 5% topical potassium permanganate saw greater than 50% reduction in ulcer size following 21 days. This demonstrates the potential of potassium permanganate to treat mild DFU but larger studies are needed (Delgado-Enciso et al., 2018).

5.5. Plant derived extracts and compounds

Medical plants extracts and compounds are often used in wound management, in particular for disinfection, debridement and the provision of a suitable environment for the natural healing process (Firdous and Sautya, 2018). A large number of surveys and studies suggest the beneficial properties of plant derived extracts on wound healing as well as on a wide range of skin diseases (Sabale et al., 2012; Sharma et al., 2013). Potentially, these plant extracts/compounds can be used alone or in combination with antibiotics to limit the infections of wound related bacteria, or to prevent opportunistic infections. They could be used prophylactically in pharmaceutical formulations for topical applications or in combination with lower doses of antibiotics, thus reducing the healing time and severity of infections, as well as the potential development of antibiotic resistance, particularly in patients with DFU.

Acyranthes aspera has been shown to possess wound healing activity due to its astringent and vasoconstrictor actions (Edwin et al., 2008). Also, honey has been shown to possess antioxidant, antibacterial and anti-inflammatory properties that promote wound healing (Yaghoobi et al., 2013). Dwivedi et al., investigated wound healing, antimicrobial and antioxidant capacity of *Pongamia Pinnata* leaf methanolic extracts in wistar rats, showing orally administered extract in rats an increased wound contraction and tensile strength, as well as TNF- α and IL-6 levels, compared to the controls. The extract exhibited antioxidant activity, as well as antimicrobial activity against gram-positive and gram-negative bacteria which further supports the early wound healing capacity of the *P. pinnata* extract (Dwivedi et al., 2017). Yaseen et al., found the quercetin-rich extract of *Ephedra ciliata* to have wound healing and anti-inflammatory potential, *via* downregulation of TNF- α *in vivo* models, and promoted wound closure in a dose- and time-dependent manner. The antimicrobial activity of the extract also suggested to contribute to wound healing (Yaseen et al., 2020).

Although various studies have shown the beneficial role of plant-derived extracts/compounds in wound management, only a small number of these studies has been focused specifically on the healing of DFUs. As previously discussed, delayed DFU wound healing is underpinned by many factors, particularly including chronic bacterial and fungal infections (Gu et al., 2013). Methicillin-resistant *Staphylococcus aureus* (MRSA), in addition to being multidrug resistant, has also emerged as a serious and common problem in patients with DFU (Kim et al., 2012). Lavender (*Urtica dioica*) and nettle (*Lavandula angustifolia*) extracts were tested against MRSA isolated from DFU, showing great antimicrobial activity against this microorganism. Positive correlation was noted between increasing the content of hydroxycinnamic acids and

1094 flavonoids (quercetin) in the extracts and decreasing bacterial growth activity (Zenão et al.,
1095 2017).

1096 Both *in vitro* and *in vivo* studies using animal models have explored the wound healing capacity
1097 of plant extracts and compounds. The tetrasaccharide stachyose from *Radix Rehmanniae* and
1098 the extract of *Radix Astragali* have been shown to significantly enhance the proliferation of
1099 human keratinocytes *in vitro* and thus could potentially contribute to wound healing. It is
1100 noteworthy, that the effect of both stachyose and the extract on the proliferation of
1101 keratinocytes was not affected by increased glucose levels, a condition that is met in patients
1102 who suffer from DFU (Ren et al., 2012). According to Alerico et al., who conducted a
1103 comprehensive *in vitro* testing of several plant species, aqueous extracts from *Achyrocline*
1104 *satureioides*, *Matricaria recutita*, *Melia azedarach* and *Mirabilis jalapa* have demonstrated the
1105 ability to stimulate keratinocyte growth (Alerico et al., 2015). Ponnusamy et al., also showed
1106 that the polyphenols rich fraction obtained from the organic extract of *Dicranopteris linearis*
1107 promotes fibroblast proliferation and migration *in vitro* (Ponnusamy et al., 2015).

1108 The aqueous extract of *C. papaya* fruit was studied for its wound healing activity in diabetic
1109 rats using excision and dead space wound models. The extract treated rats showed a 77%
1110 reduction in the wound area compared to controls which showed a 59% reduction. The *C.*
1111 *papaya* extract treated wounds were also found to epithelize faster. *C. papaya* extract also
1112 exhibited antimicrobial activity against wound related *Pseudomonas aeruginosa*, *Proteus*
1113 *mirabilis*, *Enterobacter agglumerans* and *Staphylococcus aureus* (Nayak et al., 2007).
1114 *Candida* is the most common yeast that infects diabetic ulcers and results in delaying the wound
1115 healing process (Missoni et al., 2006). The study of Kandimalla et al., investigated the anti-
1116 *Candida* and wound healing activity of the essential oil obtained from the *Cymbopogon nardus*
1117 leaves in diabetic wounds in mice (Kandimalla et al., 2016). Essential oil treatment resulted in
1118 significant reduction of *Candida* load on diabetic wounds and acceleration of wound healing,
1119 evidenced by the reduced inflammatory cytokines levels in the wounded areas compared to
1120 non-treated controls. The complete healing of the wounds was also confirmed by
1121 histopathological analysis. *Rosmarinus officinalis* (rosemary) aqueous extract and essential oil
1122 has also been found to accelerate the wound healing in diabetic BALB/c mice. The healing
1123 effect of the essential oil however, was more pronounced compared to the aqueous extract. The
1124 authors in support of the healing capacity of *Rosmarinus officinalis* found reduced
1125 inflammation, wound debridement, closure of the wound area due to rapid wound contraction,
1126 full-thickness epidermal regeneration and organization, and increase in the wet weight of

granulation tissue. Although it was not studied, the authors also attribute the healing capacity to the antimicrobial activity of extract and essential oil (Abu-Al-Basal, 2010).

There is significant evidence to support the use of plant-derived extracts and compounds in the treatment of diabetic wounds, which can indeed lead to new and exciting therapeutic strategies for DFUs. Further research is required to identify and isolate the active ingredients in the plant extracts that are involved in the wound healing processes in diabetic conditions. The application of plant-derived extracts and compounds is hindered however by the lack of clinical trials. Therefore, appropriately designed large-scale clinical trials on the use of the most promising plant extracts/compounds should to be conducted in order to fully elucidate their capacity as a DFU management tool. The application of plant extracts in combination with conventional wound management methods (e.g., wound dressings) should also be explored in depth.

6. Cold-Plasma in the treatment of DFU

Plasma medicine is a multidisciplinary field of study, combining plasma physics and chemistry, biology, and engineering. Plasma is regarded as the fourth state of matter and it can be produced by applying high voltages to specific gases resulting in the excitation, dissociation and ionisation of molecules. More specifically, plasma medicine is about producing controllable amounts of chemically reactive species that interact with biological matrices including cells and tissues (Laroussi, 2020). Plasma generators that are able to operate under atmospheric pressure and in low temperature conditions can help in the treatment of sensitive matrices including living matter (e.g. human skin/tissue) without thermal damage (Fridman et al., 2008).

Two types of plasma discharges have been mainly used in biomedical applications: the dielectric barrier discharge (DBD) and the non-equilibrium atmospheric pressure plasma jet (APPJ). Cold atmospheric pressure plasma (CAP) contains chemically reactive species including reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are known to play important biological roles. Other agents present in the plasma such as electrons and ions, UV radiation, and electric fields are also suspected to play active roles in biological applications (Laroussi, 2020; Lietz and Kushner, 2018). CAP has been proposed as a tool for various biological and medical applications for its capacity to reduce bacterial load in a wound and to initiate wound healing (Haertel et al., 2014; Weltmann et al., 2010).

Therefore, CAP could be used as a possible new treatment for patients suffering from DFUs (Heinlin et al., 2010). CAP has proven its benefits in the reduction of various bacteria and fungi in both *in vitro* and *in vivo* studies with effects becoming apparent immediately after treatment. For example, it has been shown that the generated CAP has antibacterial effects that can work against antibiotic-resistant bacteria without harming the surrounding tissue (Bourke et al., 2017; Zimmermann et al., 2012). Bacterial infection is a major contributor to the impaired and incomplete healing of DFUs. Therefore, the CAP antimicrobial effect is considered as a key factor in the application in wounds presenting with bacterial load (Haertel et al. 2014). Also, its ability to reduce the microbial load makes CAP a viable option for the potential replacement of antibiotics and the growing issue of antibiotic resistance (Petruzzi et al., 2020). Isbary et al., reported that a 2-minute CAP treatment was able to decrease the microbial load in different types of chronic ulcers, including diabetic ulcers, irrespective of bacterial species (Isbary et al., 2012).

CAP has also shown to have positive effects in chronic wound treatment. In the study of Cardinal et al., CAP treatment resulted in improved microcirculation and granulation in wound models (Cardinal et al., 2008). There are several factors, which cause impairment in diabetic wound healing process. One of the main reasons for this issue is poor blood circulation in the tissue, which leads to the lack of sufficient nutrients, and oxygen in the wound closure area (Lüscher et al., 2003). Therefore, neovascularization is one of the most important factors contributing to diabetic wound healing. According to Fathollah et al., CAP treatment of wounds in diabetic rats for 10 min induced neovascularization 7 days post treatment (Fathollah et al., 2016), with new epidermal layers being formed 3 days post treatment in the diabetic rats. Hirata et al., also reported plasma treatment resulted in angiogenesis in burn wounds, hypothesised to result from the reactive agents in plasma inducing the release of growth factors (Hirata et al., 2014). Indeed, it has now been revealed that plasma enhances the wound-healing rate in diabetic rodents, presumably due to increased levels of TGF- β 1, superoxide dismutase, glutathione peroxidase and catalase (Cheng et al., 2018; Fathollah et al., 2016).

There is only a small number of clinical trials done so far on the effects of cold plasma on DFU. Stratmann et al. (2020) conducted a placebo-controlled, patient-blinded study to assess the effect of CAP, generated using argon, in addition to standard care treatment compared with placebo on DFU healing. CAP treatment resulted in a significant increase in wound healing, both in total mean area reduction and mean time to relevant wound area reduction. Interestingly, reduction of infection and wound microbial load was not significantly different

between CAP and the placebo treatment. These results suggested that CAP led to accelerated wound healing reaching the 10% and the 20% surface reduction mark earlier compared to the placebo treatment irrespective of infection and bacterial load reduction and infection status. Mirpour et al., conducted a randomized double-blinded clinical trial for the use of CAP in DFU. They used helium as the inducer gas to produce cold plasma and their results showed that that three sessions of CAP treatment (for 5 min) per week, for three consecutive weeks, accelerate wound healing in DFU. Specifically, CAP treatment effectively reduced the wound size and also resulted in an immediate but brief reduction of wound bacterial load. No correlation for bacterial load and wound size was found (Mirpour et al., 2020).

The application of CAP in biomedicine opens up many new possibilities. Although, new fundamental knowledge is continually being developed in this area, there are still unanswered questions. Based on the information presented here, there is clear potential for CAP to be used for DFU treatment, as it is able to both reduce the wound microbial load and accelerate wound healing. It is important to note that potential differences in the antibacterial effects and healing times are to be expected between studies, as these will be dependent on the treatment time, the plasma generator system, working gas composition, distance to plasma source, different environmental variables, and the characteristics of the tissue/wound being treated. Another important factor that needs to be considered in the future is how CAP treatments is tolerated amongst patients with DFUs.

7. Future directions of DFU

Due to the increasing incidence of both DM and DFU globally, as well as predicted increases in market growth to meet patient demand, more advanced treatments for DFU wound healing will be required in order to cope with clinical demand.

3D bioprinting has the potential to revolutionise the treatment of DFU. The ability of this technique to rapidly produce complex scaffolds, which can incorporate effective therapeutics or cells, could potentially eliminate the need for some traditional therapies to be implemented, reducing both costs and time involved in treating DFU. Moreover, the development towards *in situ* bioprinting provides the additional benefit of personalised treatment where other scaffold fabrication techniques do not.

1221 This alone however will not be sufficient. A greater focus on preventative methods is required,
1222 something which is currently underrepresented in literature. Nonetheless, by tackling these
1223 issues and incorporating 3D bioprinting as a key tool in treating DFU, we believe it is possible
1224 to lower the rate of amputations associated with DFU as well as lowering the economic burden.

1225 In this section, we will offer some of our views into how greater focus on DFU prevention and
1226 3D bioprinting innovations in particular could act to improve the care of DFU in the near future.

1227 7.1. Personalised treatment

1228 The use of personalised medicine is expected to increase greatly over the coming years, due to
1229 the substantial benefits that this provides.

1230 3D bioprinting allows scaffolds of various structural geometries and sizes to be easily modified
1231 in order to be moulded to patient wound topology on an individual level, for example. This
1232 would allow complete contact with and coverage of the wound to maximise the potential for
1233 successful wound healing outcomes. One technique incorporating this approach in *in situ*
1234 bioprinting, which is gaining considerable interest and has shown feasibility of producing
1235 bioinks suitable for this application.

1236 Albanna et al., developed a mobile *in situ* bioprinting system with built in imaging system to
1237 provide delivery of fibrin/collagen hydrogel laden with keratinocytes and fibroblasts. This
1238 technique allowed the scaffolds to be tailored to each patient based on individual wound
1239 topology, showing successful wound healing characterised by extensive collagen deposition
1240 and well-developed vasculature (Albanna et al., 2019). Moeinzadeh et al., developed
1241 injectable, *in-situ* forming collagen/alginate hydrogels suitable for the delivery of cells and
1242 growth factors for potential use as a bioink in bioprinting applications. Pre-crosslinking
1243 maintained stability following injection. This overcomes printability issues that are associated
1244 with collagen and produced a scaffold which retained 90% cell viability for 7 days following
1245 injection, lead to healing of bone defects in a rat model within 8 weeks through release of
1246 bovine serum albumin (BSA) and bone morphogenetic protein 2 (BMP2) (Moeinzadeh et al.,
1247 2021).

1248 O'Connell et al., demonstrated rapid crosslinking (less than 1 second) of a coaxial bioink
1249 directly following extrusion. At an optimal formulation of GelMA 10%, gelatin 3% and HA

1%, this overcomes printability and gelation issues observed with some pre- and post-crosslinking methods in bioprinting (Connell et al., 2020).

7.2. Prevention

With increasing prevalence of diabetes globally and the expectant increase in DFU, prevention will play a key role in both limiting the costs of treating DFU as well as reducing clinical burden on healthcare systems. It has been estimated that up to 75% of DFU are preventable (Bus, 2016).

Currently, many existing barriers are preventing effective diagnosis and treatment of DFU. Despite an increase in knowledge and awareness in how to prevent and manage DFU over the years (Desalu et al., 2011), recent studies suggest there is room for considerable improvement in patients compliance with these practices (Abdulghani et al., 2018). One way to tackle this is through greater disease awareness and patient education. However, these need to be studied on a larger scale and in various countries to accurately reflect global attitudes towards DFU prevention.

There is also the need for greater research into prevention of DFU. Although extensive research has been conducted into treatment options for DFU, prevention strategies, and how these can be translated into clinical practice, remain underexposed in research. Currently, for every one randomised control trial investigating DFU prevention, ten exist on DFU treatment (Bus, 2016).

8. Conclusions

The incidence of DFU is expected to increase globally, in correlation with an expected increase of diabetes. In order to meet patient demand, new innovative treatment options are required to overcome the limitations of existing treatments. Current evidence suggests the effectiveness of 3D bioprinted scaffolds in the treatment of DFU due to their ability to achieve high cell densities and rapid production of complex structures of various geometries. Furthermore, the progression of bioprinting capabilities towards *in situ* bioprinting presents exciting opportunities for future treatment options in diabetic wound healing, increasing the prospect of personalised treatment, which would help to revolutionise current treatment options. These developments, combined with increased research focus and patient compliance with critical

1279 prevention strategies are expected to reduce clinical need and improve current diabetic wound
1280 healing outcomes.

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Figures

Figure 1. Overview of the normal wound healing process (left) *versus* the impaired diabetic wound healing process (right); reprinted from (Spampinato et al., 2020), with permission.

2160 **Figure 2.** Schematic representation of single electrospinning apparatus setup; reprinted from
2161 (Chen et al., 2019), with permission.

2164 **Figure 3.** Schematic representation of FDM; reprinted from (Gebisa and Lemu, 2018), with
 2165 permission.

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2167 **Figure 4.** Schematic illustration of (a) continuous inkjet (CIJ) and drop-on-demand (DOD)
 2168 printing systems using (b) thermal and (c) piezoelectric technology; reprinted from (Alamán et
 2169 al., 2016), with permission.

2172 **Figure 5.** Schematic representation of extrusion-based bioprinting technologies; reprinted from
2173 (Kryou et al., 2019), with permission.

Figure 6. Schematic representation of laser-assisted bioprinting, where (a) show a transparent glass, (b) illustrates the thin metal layer and (c) the vaporization-induced bubble; reprinted from (Kačarević et al., 2018), with permission.

Figure 7. Stress-strain curves of sodium alginate films after dipped into variety of concentrations of CaCl_2 for 2 and 8 min; reprinted from (Fadhilah et al., 2019), with permission.

Figure 8. Graphic representation of PLGA-alginate bilayer membrane; reprinted from (Wang et al 2019), with permission.

Figure 9. Schematic representation of an atmospheric-pressure plasma jet system (APPJ). The APPJ consists of a quartz tube. Copper ring electrodes are located on the outside of the tube separated by 10 mm and with the ground electrode located 10 mm from the jet exit. gAS admixture flows through the tube and is excited by a high-voltage amplifier; reprinted from (Nicol et al., 2020), with permission.

2197 **Table 1:** Standard therapies used to treat DFU

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Method	Description	References
Offloading	<p>Non-removable knee-high total contact cast (TTC) devices are the gold standard option for neuropathic plantar or midfoot DFU.</p> <p>TTCs have been shown to greatly reduce the time for DFU healing when compared to conventional dressings.</p> <p>Non-removable devices are more effective than removable devices.</p> <p>Low risk of venous thrombo-embolism (VTE) associated with TCC use.</p>	(Bus et al., 2020; King et al., 2017; Morona et al., 2013; Sahu et al., 2018; Sudhir et al., 2020; Tonge et al., 2019)
Glycaemic control	<p>Lower HbA1C levels are associated with faster healing rates. HbA1C levels between 7 – 8% provide benefit to the wound healing of DFU.</p> <p>Negative correlation has been reported between 1.0% increases in HbA1C levels and the rate of DFU wound healing.</p> <p>Raised HbA1C has been associated with improved wound healing in patients where baseline HbA1C was below 7.5%.</p>	(Christman et al., 2011; Dhatariya et al., 2018; Fesseha et al., 2018; Gorantla et al., 2020; Kumar et al., 2016; Xiang et al., 2019)
Debridement	<p>Debridement is the removal of necrotic and senescent tissues as well as foreign and infected materials from a wound.</p> <p>Surgical debridement: large-scale removable of both non-viable tissue and immediately-surrounding viable tissue.</p> <p>Biological debridement: maggot debridement therapy accelerates wound healing. Genetically engineered maggots can be used to secrete human platelet derived growth factor.</p> <p>Mechanical debridement: application of a wet gauze which then adheres to the skin and, upon its removal, results in non-selective removal of dead tissue surrounding the wound.</p> <p>Enzymatic debridement: uses natural proteolytic enzymes to remove non-viable tissue in a selective manner. Clostridial collagenase ointment (CCO) is commonly used to successfully treat neuropathic non-ischemic DFU.</p>	(Ananian, 2020; Azad et al., 2016; Healthline, 2019; Jimenez et al., 2017; Linger et al., 2016; Motley et al., 2015; Tardivo et al., 2017; Wound Source, 2019)
Infection management	<p>Approximately 40% of DFU become infected.</p> <p>Before treatment commences microorganisms involved need to be identified and infection severity classified.</p> <p>Mild infection: Oral administration of appropriate antibiotic for up to seven days.</p> <p>Moderate infection: Antibiotic administered either orally or intravenously, for greater than seven days, or for six weeks in the case of osteomyelitis.</p> <p>Severe infection: antibiotic combination treatment.</p>	(Bus et al., 2019; Jia et al., 2017; National Institute for Health and Care Excellence (NICE), 2015)

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2200 **Table 2:** Advanced therapies used to treat DFU

Method	Description	References
Wound dressing	<p>Hydrogel dressings, amorphous topical applications which adhere to the site of injury, are used most commonly.</p> <p>Hydrogels provide the benefit of tuneable properties, such as porosity, whilst also mimicking the natural granulation tissue which promotes biocompatibility.</p> <p>Hydrogel dressings are more effective than hydrofiber dressings.</p> <p>Hydrogel dressings can be laden with cells and growth factors. Large burst release phases can be observed, reducing their viability in the treatment of chronic ulceration where sustained drug release would be more effective.</p>	(Chai et al., 2017; Chen et al., 2015; Hajimiri et al., 2016; Motley et al., 2015)
Negative pressure wound therapy (NPWT)	<p>NPWT promotes healing of acute and chronic wounds through use of negative pressure in a closed system.</p> <p>Decreases mRNA expression of IL-1B, IL-6, TNF-α, iNOS and MMP-9, while upregulating VEGF and TGF-B1 expression.</p> <p>Improves blood flow and micro-vessel maturation in diabetic wounds and promotes a moist wound environment, which is favourable for healing.</p> <p>NPWT is not more effective in the treatment of DFU compared to moist wound dressings.</p> <p>Limited effect on the reducing amputations.</p>	(Abdullah Al-Mallah, 2018; Jung et al., 2016; Junker et al., 2013; Karam et al., 2018; Ma et al., 2017; Muhammad et al., 2015; Seidel et al., 2020; T. Wang et al., 2019)
Skin substitutes	<p>Skin grafts such FDA-approved Dermagraft® have shown improved healing of chronic wounds, without adverse events.</p> <p>The potential of this method has been demonstrated through acellular membranes bioengineered with collagen, proteoglycans and glycoproteins, resulting in delayed onset of rejection <i>in vivo</i>.</p> <p>SIKVAV (Ser-Ile-Lys-Val-Ala-Val) peptide-modified chitosan hydrogel displayed more rapid wound healing, with greater collagen deposition, myofibroblast deposition and angiogenesis, compared to positive and negative controls.</p>	(Chen et al., 2018; Marston et al., 2003; Stubenitsky et al., 2009)

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2203 **Table 3:** Advantages and disadvantages of electrospinning

Method	Advantages	Disadvantages	References
Electrospinning	High porosity structures Tuneable pore size Good reproducibility High drug loading Wide variety of materials Nanoscale fibers Allows well distributed cellular attachment Can be used alongside 3D printing Cost-effective and easily scaled-up Coaxial methods can improve mechanical properties of the scaffold	Additional processing steps such as crosslinking may be required to achieve scaffolds with sufficient mechanical strength Highly volatile solvents may be required during production	(Junker et al., 2013; Mohammadi et al., 2016; Ren et al., 2018; Samadian et al., 2020; Surucu and Turkoglu Sasmazel, 2016)

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Table 4: Advantages and disadvantages of fused deposition modelling

Method	Advantages	Disadvantages	References
Fused deposition modelling (FDM)	Produces scaffolds of various designs and geometries Easy modification of scaffold designs Rapid production (minutes to hours) Easily scaled-up Can produce scaffolds with good mechanical properties, no cytotoxicity and which promote cellular attachment and proliferation Can print high viscosity solutions No solvents required Can achieve better mechanical properties than electrospinning Production of large scaffolds Can be used in combination with other techniques such as gas foaming	Scaffolds produced by this method may not be suitable for tissue engineering purposes due to their limited flexibility Requires high shear, high temperature and high pressures are often required May require additional steps such as crosslinking of the biomaterial	(Domingos et al., 2009; Gao et al., 2020; Guo et al., 2009; Intini et al., 2018; Kovalcik et al., 2020; Song et al., 2018; Trachtenberg et al., 2016)

2209 **Table 5:** Advantages and disadvantages of bioprinting techniques.

Method	Advantages	Disadvantages	References
Inkjet-based bioprinting	Low-cost method High cell loading and viability Control of droplet size High resolutions achieved (100µm) Easily scaled-up Has been used for <i>in situ</i> bioprinting purposes Direct printing of cells and growth factors	May require additional processing steps such as crosslinking of the bioink Polymer degradation has been associated with continuous inkjet bioprinting	(Albanna et al., 2019; Haas et al., 2017; Negro et al., 2018; Takagi et al., 2019; Wheeler et al., 2014; Yanez et al., 2015; Zhang et al., 2017)
Extrusion-based bioprinting	Most suitable bioprinting method for large scaffolds and soft tissue purposes Low-cost method Moderate (300µm - 600µm) to high (200µm) resolution High seeding capacities Suitable for high viscosity materials Low printing temperatures and pressures in some cases Direct printing of cells and growth factors	Printing of high viscosity materials will require faster printing speeds, thus negatively affecting cell viability May require additional steps such as crosslinking of the bioink	(Cubo et al., 2016; Fakhruddin et al., 2018; Frost et al., 2019; Kolesky et al., 2016; McCormack et al., 2020; Soltan et al., 2019; Tan et al., 2016; Visser et al., 2013; Zhuang et al., 2019)
Laser-assisted bioprinting	No printing nozzle required High speed printing High resolutions High cell densities Has been used for <i>in situ</i> bioprinting purposes	Time-consuming preparation Expensive Laser is a potential disruption to cell viability	(Guillotin et al., 2010; Keriquel et al., 2017; Kérourédan et al., 2019b)

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2212 **Table 6:** Natural polymers for 3D bioprinted scaffolds

Material	Advantages	Disadvantages	References
Collagen	Highly biocompatible and non-toxic pH neutralisation at a temperature of 37°C is required for gelation of collagen (gelation occurs at a low temperature i.e. no high temperatures are required which could affect cell viability) Highly permeable	Poor mechanical properties Long gelation times (~30 minutes) Commonly crosslinked using chemical and physical methods Low viscosity	(Bou-Akl et al., 2013; Carolina et al., 2020; Davidenko et al., 2015; Dong and Lv, 2016; Heo et al., 2016; Kozłowska et al., 2017; Skardal and Atala, 2015; Tian et al., 2016; Zhou et al., 2018)
Gelatin	High availability at low cost Low immunogenicity and high biocompatibility Structurally similar to collagen Promotes cell adhesion and proliferation Good injectability	Native gelatin has reversible temperature-dependent sol-gel transition Lack of thermal stability Faster degradation rates Poor mechanical properties	(Augustine et al., 2021a; Bello et al., 2020; Cheng et al., 2019)
Alginate	Non-toxic and biocompatible Easily modified Maintains moist wound environment	Poor mechanical properties Exhibits shear-thinning behaviour Requires chemical crosslinking to form hydrogel	(Chuang et al., 2017; Fadhilah et al., 2019; Gwon et al., 2015; H. Li et al., 2016; Thomas et al., 2020)
Fibrin	Promotes cell adhesion Biocompatible Excellent wound healing ability	Long gelation time (~30 minutes) High viscosity can lead to poor extrusion Poor mechanical properties and rapid degradation Poor shape fidelity	(Climov et al., 2016; Cubo et al., 2016; de Melo et al., 2020; Losi et al., 2013; Robinson et al., 2017)

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2215 **Table 7** Rheological data of sodium alginate films that immersed in different concentrations
 2216 of CaCl_2 (Fadhilah et al., 2019).

Molarity CaCl_2 (M)	Period (min)	σ (MPa)	ε (%)	YM (MPa)
0.2	2	1.00 ± 0.2	15.21 ± 1.1	8.26 ± 2.3
	8	1.05 ± 0.2	15.19 ± 1.5	9.23 ± 0.6
0.4	2	1.25 ± 0.2	16.90 ± 2.5	9.64 ± 2.0
	8	2.43 ± 0.2	16.25 ± 1.0	20.96 ± 10.6
0.6	2	1.82 ± 0.2	12.22 ± 3.2	17.88 ± 6.1
	8	2.90 ± 0.5	18.63 ± 3.1	24.26 ± 12.1
0.8	2	3.14 ± 1.2	18.83 ± 3.0	24.97 ± 10.2
	8	3.92 ± 0.3	21.08 ± 1.3	27.81 ± 7.5

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2219 **Table 8:** Synthetic polymers for 3D bioprinted scaffolds.

Material	Advantages	Disadvantages	References
Poly(lactic-co-glycolic acid)	Biocompatible Low-cost and wide availability Good mechanical properties Degradation rate can be easily modified	Higher temperatures and pressures required for printing compared to natural polymers	(Allevi, 2020; Keles et al., 2015; S. Wang et al., 2019)
Polycaprolactone	Ease of processing due to low melting point and gelation temperatures Slow rate of degradation Non-toxic and biocompatible Good mechanical properties	Requires high printing temperatures and pressures due to high viscosity Longer printing times due to high viscosity Hydrophobic nature can affect cell adhesion	(Azimi et al., 2014; Burns et al., 2010; Cellink, 2020; Nair et al., 2017; Sukanya and Mohanan, 2018; Wang et al., 2016)
Polyurethane	Good mechanical properties Good elasticity Biocompatible Excellent printability at low temperatures Promotes cell adhesion and proliferation	Often requires additional crosslinking stage during processing	(Hsieh and Hsu, 2019; Tsai et al., 2015)
Polyethylene glycol	Promising wound healing capabilities when used in combination with sodium alginate Promotes M2 macrophage polarisation Good mechanical properties Easily modified	PEG requires additional processing step such as modification to PEDGA or addition of other supporting biomaterials, increasing costs and production time	(Asawa et al., 2018; Cheng and Chen, 2017; Ilhan et al., 2020)

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