1-5-2017

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**Citation**

Innovations in Gene and Growth Factor delivery systems for Diabetic Wound Healing

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/term.2443

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Abstract:

The rise in lower extremity amputations (LEAs) due to non-healing of foot ulcers in diabetic patients calls for rapid improvement in effective treatment regimens. Administration of growth factors (GFs) are thought to offer an off-the-shelf treatment however, the dose- and time-dependent efficacy of the GFs together with the hostile environment of diabetic wound beds impose a major hindrance in the selection of an ideal route for GFs delivery. As an alternative, the delivery of therapeutic genes using viral and non-viral vectors, capable of transiently expressing the genes until the recovery of the wounded tissue offers promise. The development of implantable biomaterial dressings capable of modulating the release of either single or combinatorial GFs/genes may offer solutions to this overgrowing problem. This article reviews the state of the art on genes and proteins delivery and the strategic optimization of clinically adopted delivery strategies for the healing of diabetic wounds.

Keywords: Diabetic foot ulcer, wound healing, growth factors, gene, biomaterials

1. Introduction:

Diabetes has become an ever escalating global health crisis. Rapid urbanization, transition in nutritional status and increasing sedentary lifestyles is often mentioned the cause for dynamic rise in the epidemic (Hu, 2011). In 2014, 387 million people was estimated to suffer from diabetes and it accounted for 11% of global health expenditures or at least $612 billion (da Rocha Fernandes et al., 2016). A study reported recently in Diabetes Research and Clinical Practice, estimated that by 2035, the number of diabetes affected people would reach at least 592 million people worldwide, with two-thirds of all diabetes cases occurring in low- to middle- income countries (Guariguata et al., 2014). Given such statistics and with no curative solution, diabetes poses serious threat to the economies of both developed and developing nations.
The expenses involved for the treatment of multiorgan dysfunction resulting from vascular (macro and micro) complications in diabetic patients could be held responsible for the huge burden imposed upon the global economy (American Diabetes Association, 2013). Chronic hyperglycemia is known to be the main factor for the initiation of diabetes-associated vascular complications. Under such pathological conditions, an injury to the skin could cause serious life-threatening risk due to the loss of innate healing mechanism of the skin. Diabetic patients with foot wounds often fall the victim to such risk. Histopathological features of wounds in diabetic foot are identified by abnormal microvessels that can be cuffed with collagen, laminin, fibronectin, or fibrin in the wound edges (Luis Herrera, 2013). Further accumulation of debris can exacerbate the development of ulceration due to increased pressure at the wound edges, a feature predominantly observed in diabetic neuropathy, where an individual loses protective pedal sensation (Krishnan et al., 2007). This population is prone to develop chronic non-healing diabetic foot ulcers (DFUs), which are estimated to occur in 15% of all persons with diabetes. DFU precede 85% of all diabetes related LEAs and presents as a significant mortality risk factor (Hoffstad et al., 2015, Snyder and Hanft, 2009).

Timely prevention and healing of diabetic ulcerations form the baseline for amputation prevention and reduction in mortality rate. Currently, debridement in conjunction with infection control, off-loading to relieve pressure, and maintenance of a moist wound bed has been the standard wound-care practice for DFUs. Continual deployment of the multidisciplinary wound-care setting is considered favorable for healing by converting chronic wounds into acute, but the consistently poor vascular status necessitates longer hospitalization stays, incurring high expenditure owing to incremental resource utilization, including hospitalization charges (Driver et al., 2010). Despite high initial costs, revascularization using advanced treatment modalities such as growth factors (GFs), hyperbaric oxygen therapy (HBOT) and bioengineered skin grafts have gained a faster pace
than conventional wound care in achieving faster wound closure and reducing ulcer recurrence, eventually improving the overall quality of life of DFU patients (Snyder and Hanft, 2009). However, the optimal treatment regimen is subject of much debate. One reason for this is that, the involvement of diverse mechanisms in impairing physiological regulations in the diabetic wound bed presents a barrier in elucidating the effective route for treatment. Clearly however, considering the urgent treatment need, therapies targeted to improving the angiogenic pathways have significant potential in stimulating vascularization and accelerating healing. Here, we aim to provide insight into in vivo delivery of biomolecular therapeutics, particularly growth factors (GFs) and discuss their efficacy in activating the angiogenic pathways for timely healing of wounds in diabetic patients.

1.1 Impaired angiogenesis in diabetic wounds:

Angiogenesis is characterised by the sprouting of new blood vessels from pre-existing vessels and is a critical step in wound healing as it allows provision of oxygen and nutrients via the blood streams to inhibit apoptosis of vital cells in the injured tissue. Immediately, in response to tissue injury, a typical angiogenesis follows a complex multistep process involving extracellular matrix (ECM) degradation, proliferation, survival, migration and morphological changes of endothelial cells (ECs) and their anastomosis to assemble into a mature vasculature, which encompasses concurrently with overlapping wound healing phases of coagulation, homeostasis, inflammation and proliferation with matrix deposition and remodeling (Li et al., 2003, Velnar T, 2009). A schematic of a typical wound healing process is presented in Figure 1. In chronic wounds, the progressive synchrony of the healing phases is lost and the process of angiogenesis remains stalled in the inflammation or proliferation phase leading to the formation of impaired granulation tissue (Falanga, 2005). Briefly, angiogenesis in its inflammation phase commences with the production of nitric oxide (NO)
by inflammatory cells (e.g. macrophages) which in turn stimulates vasodilation and permeability, facilitating immune cells (e.g. neutrophils) extravasation and release of pro-inflammatory cytokines and chemokines, which further activates neighbouring ECs and fibroblasts to release angiogenic factors. The inflammatory cells themselves would release angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β), Angiopoetin (Ang) and cytokines such as tumor necrosis factor-α (TNF-α), that can stimulate angiogenesis following accumulation at the site of inflammation.

Elevation in hypoxia is another feature in inflammation which is characterized by the production of hypoxia inducing factors (HIFs), which further promotes transcription of angiogenic genes such as VEGF and Ang-2 (Costa et al., 2007, Polverini, 2012). However, in hyperglycemia, these mechanistic events of inflammation-mediated angiogenesis are interfered upon by accelerated accumulation of advanced glycation end products (AGEs), whose formation as a result of non-enzymatic glycation of proteins, lipids and nucleic acids may occur both extra- or intra-cellularly (Singh et al., 2014).

Within the microvasculature, AGEs cause aberrant crosslinking of the ECM proteins (such as collagen) altering their binding affinity for proteoglycans and sensitivity to collagenase. This event in turn, drives the generation of reactive oxygen species (ROS) which interferes with the production of NO by endothelial NO synthase. On the other end, AGEs induce the expression of their receptors (RAGEs) on the surface of various cell types, whose interaction with the circulating AGEs activates a myriad of abnormal intracellular signalings (mainly the nuclear factor-kappa B pathway), perpetuating pro-inflammatory and matrix metalloproteinases (MMPs) activities, and elevation of oxidative stress, further contributing to AGEs deposition (Pierce, 2001, Stirban et al., 2014). While this pathological loop is at
play, GFs sequestration to the ECM is altered and the abnormal elevation in proteolytic activity degrades the GFs substantially, resulting in local depletion of GFs, inhibiting angiogenesis and healing (depicted in Figure 2) (Briquez et al., 2015). Furthermore, accumulation of AGEs is believed to cause segmental demyelination in the nerves leading to interruption in axonal transport and alter the expression of neuronal peptides such as nerve growth factor (NGF) (Apfel et al., 1998). The resulting dysfunctioning of sensory nerve fibers is known to compromise the immunomodulation of the skin during the inflammatory phase of wound healing (Pradhan et al., 2009). Hyperglycemia also has an adverse effect on the degree of erythrocyte aggregation and deformability, and haemorheology thereby impeding blood flow to the distal ends of the wound, limiting the supply of oxygen (prolonging hypoxia) and nutrients for tissue homeostasis (Cho et al., 2008). Collectively, these events imply a portal for supplementation of exogenous GFs, which in turn would stabilize angiogenesis and induce normal healing in diabetic wounds.

2. Growth factor-therapy for diabetic wound healing:

Applications of exogenous GFs are considered a promising approach for the treatment of most chronic or degenerative disorders. The rationale for using GFs has been attributed to its ability to trigger and co-ordinate a myriad of cellular and molecular events, which is critical for successful healing of injured tissues (Barrientos et al., 2008). A range of GFs including platelet derived growth factor (PDGF), VEGF, EGF, FGF, TGF, keratinocyte growth factor (KGF), insulin-like growth factor (IGF) and HIF have been well documented in reviews for their potential in accelerating the wound healing process (Bennett et al., 2003). Of these, PDGF, VEGF, EGF, FGF, TGF-β1 have been applied in clinical trials for the treatment of DFU (Martí-Carvajal et al., 2015).
Clearly, nerve fiber loss in diabetic neuropathic patients is a well known factor that contributes to impaired healing of diabetic wounds, but the understanding on the influence of neuropathic condition in impaired healing still remains warranted. Nevertheless, topical application of NGF formulations into diabetic wounds have been demonstrated to promote regeneration of nerves and induction of reparative angiogenesis mediated by recruitment of ECs to the wound site (Graiani et al., 2004, Muangman et al., 2004). Moreover, Thomson et al. in 2010, using a non-human primate model, demonstrated for the first time that connective tissue growth factor (CTGF) was also dysregulated in diabetic wounds (S. E. Thomson, 2010). Later in 2015, their group reported that topical application of CTGF could promote healing through re-epithelialization in diabetic rodents (Henshaw et al., 2015).

2.1. Growth factor delivery routes adopted in clinical studies for DFU:

Early clinical trials with GFs for DFU dates back to mid-1990s with that of Becaplermin (Regranex®), a topical gel formulation of recombinant human (rh) PDGF-BB. It is the first GF approved by the US Food and Drug Administration (FDA) for use in DFUs. However, its progression to actual clinical practice remains unmet, as reported efficacies were based on small randomized trials which were performed under well controlled environments, failing to yield evidence-based guidelines specifying the time and required mode of treatment. Furthermore, the difficulty with routine clinical experience and less successful outcomes have prompted the search for an alternative mode of delivery that can prolong the exposure of Becaplermin alone or in combination with other GFs to the wound site (Papanas and Maltezos, 2008). Additionally, applicability of topical gel formulation becomes limited with increasing severity of ulcers as the GFs undergo proteolytic breakdown leading to inadequate diffusion to the deeper wound layers. With the development of topical spray form, the above limitation appears to have been compensated to a certain extent, as it allows early maximal
contact of the GFs with the entire wound surface. The therapeutic efficacy was demonstrated in a clinical study where topical spraying of rhEGF (Easyef®) twice daily resulted in wound size reduction over 80% by eighth week irrespective of the grade of ulcer (Tuyet et al., 2009).

Alternatively, intralesional injection offers a relatively painful but more localized route for GF delivery. Acosta et al. in 2006, performed the proof-of-concept trial and showed that intralesional injection of rhEGF into DFUs of Wagner’s Grade 3 or 4 (ulcer area > 20 cm²) thrice weekly, received appreciable granulation response and wound closure rate with enhanced angiogenesis (Acosta et al., 2006). Their study was further validated in multi-centre and placebo-controlled trials (Fernández-Montequín et al., 2007, Fernández-Montequín et al., 2009). Taken together, these clinical studies are representative of the feasibility of an easy in vivo approach (injection or topical) for therapeutic delivery to diabetic wounds. However, serious concerns exists that might interfere with the benefit-risk balance of the treatment regime, and halt the progression from bench to bedside. For example, there is dose-dependent (more than three tubes) risk of cancer stimulation with Becaplermin application (Papanas and Maltezos, 2010). On the other hand, topical spraying or intralesional injection requires frequent disruption of the dressing, that is essentially used to occlude the wound from transference of micro-organisms from other external environmental sources (Mertz et al., 1985). Although the level of risk of infection with the above practice is unclear, termination of treatment resulting from infection have been reported (Fernández-Montequín et al., 2007, Tuyet et al., 2009).
Achieving favourable benefit-risk balance remains the key to clinical translation. The need to overcome the limitations described above has led to exploring into novel GF delivery systems/techniques that serve to protect the GFs from degradation but at the same time allow controllable release and reduce the frequency of administration (Gainza et al., 2015). Alternatively, gene delivery approaches which utilize deoxyribonucleic acid (DNA) encoding for therapeutic genes could potentially provide a more stable and effective approach to allow sustained and controlled release of therapeutic factors (O'Brien, 2011). The following sections will discuss the various approaches adopted for the delivery of GFs and genes, as applied to diabetic wound healing.

3. Biomaterial systems as depots for therapeutics delivery to diabetic wounds:
The growing interest in the development of biomaterial systems for therapeutic delivery to diabetic wounds can be attributed to their ability to sequester and release clinically significant doses of the therapeutics for an extended period of time within the targeted site. Some of the existing challenges with designing biomaterials for such applications include the maintenance of GF structure and bioactivity during fabrication, high encapsulation efficiency, bioavailability and achieving complete release with a therapeutically active pharmacokinetic profile from the biomaterial depot. Understanding the time- and dose-dependent response to individual GFs is also important. For example, it is acknowledged that VEGF-A requires a higher initial release for initiation of angiogenesis, followed by steady but lower release rate maintained within the therapeutic window, while EGF requires prolonged exposure to be effective (Amsden, 2015). In line with this, the feasibility of designing a novel biomaterial depot with the ability to tailor the release kinetics of two distinct GFs, corresponding to their effects on stabilizing angiogenesis has been well demonstrated as far back as 2001. This study also stands as a notable example that bolus delivery of multiple GFs is not sufficient to sustain angiogenesis (Richardson et al., 2001). Additionally, the fact that GFs have short
half-life, limited diffusion lengths, and very low concentration (10^9 to 10^{11} M)-associated bioactivity necessitates the persistent presence of the biomaterial depot within or in the implanted site for extended time frames (from days to weeks) without inducing host-immune response. This condition primarily requires that the biomaterials be highly biocompatible however, biomaterials that can be degraded and excreted or resorbed into host tissues are gaining utmost importance for the development of implantable systems.

The use of degradable biomaterials eliminates the need for a second surgical intervention for implant removal, but also allows for improved healing by facilitating tissue ingrowth into the degrading construct. As the degradation is believed to be mediated by specific biological activity, the process is generally termed biodegradation. Currently, the commonly used biodegradable biomaterials for therapeutic applications include synthetic polymers of polyester family such as PGA, PLA, poly(lactic-co-glycolic acid) (PLGA), polycaprolactone (PCL) and/or polymers of natural origin namely, collagen, gelatin, fibrin, hyaluronic acid, dextran, alginate, and chitosan. The synthetic polymers are known to biodegrade mainly via cleavage of hydrolytically sensitive ester bonds in the polymer while that of natural polymers are often enzymatic. The erosion behaviour affects therapeutic release kinetics in a biomaterial making biodegradability a fundamental part of biomaterial implants (Amsden, 2015, Nair and Laurencin, 2007). However, due to the variations in site-to-site and pathological conditions in patients, the biomaterial system may need to be uniquely tailored for controlled degradation in situ (Nair and Laurencin, 2007). Developing a biomaterial-based delivery systems with predictable degradation kinetics will enable one to control the release profile of therapeutics, resulting in localization of optimized concentrations of therapeutics (Lee et al., 2011). Typically, the degradation kinetics of the biomaterial systems are determined in vitro by incubating it in either phosphate buffered saline (PBS) or
simulated body fluid (SBF) solutions. Although in many cases, the results of *in vitro* have not been reflective *in vivo* (Bölgen et al., 2005, Lu et al., 2000). Degradation studies conducted in an environment that closely mimics the *in vivo* environment would give valuable insights for the development of more precisely controllable biomaterial-based delivery systems. For instance, knowledge on the physicochemical properties of the site of implantation and its duration of contact with the tissues and body fluids could help choose the optimum incubation media and the duration of study (Azevedo and Reis, 2005). Keeping note of the above highlights, herein we discuss the different forms of therapeutic biomaterial systems and its efficacy in the treatment of diabetic wounds.

3.1 Particulate systems for sustained release of GFs into diabetic wounds:

Biodegradable polymeric particles are one of the most explored delivery systems for site-specific controlled release of therapeutics. The emulsion/solvent extraction methods are the most commonly employed to prepare polymeric particles of various sizes (with the range of 1-1000 μm for microparticles and <1 μm for nanoparticles (Zhang and Uludağ, 2009)). Due to the high solubility of GFs in water, the emulsification process is designed to yield a polymeric system with GFs embedded in the hydrophilic phase while the hydrophobic phase assembles as the protective shell/layer. Typically, the GFs release curve from these systems exhibits a biphasic pattern with an initial burst release followed by a gradual and sustained release. But, an improved control over the release rate is deemed achievable by developing systems with pre-defined diameter or shell thickness. Often, smaller particles are known to exhibit faster initial release of therapeutics than larger particles. This behaviour by smaller particles is thought to result from a combined effect of early diffusion of therapeutics owing to the shorter penetration length of water to the center of particle and higher surface-area to volume ratio facilitating increased efflux of therapeutics (Kim and Pack, 2006).
Clearly, optimization of the fabrication parameters to yield reproducible particles of desired size will contribute significantly in the field of polymeric particles-based delivery systems.

A double-emulsion method was employed to develop rhEGF loaded PLGA nanoparticles. The particles exhibited an encapsulation efficiency of 85.6% but a short release period lasting only up to 24 hours in vitro. The rhEGF-PLGA nanoparticles were sprayed once daily into the wound of diabetic mice but failed to induce any healing-response until the third day of treatment. However, within 7-21 days of study, the group treated with rhEGF-PLGA nanoparticles exhibited the fastest healing rate as compared to groups treated with rhEGF or PLGA alone. The accelerated healing was attributed to the maintenance of an effective local concentration of bioactive rhEGF released from the PLGA nanoparticles (Chu et al., 2010). A study by Cherddy et al adopted the intradermal route for the delivery of VEGF-loaded PLGA nanoparticles. In vitro study found that the PLGA nanoparticles could sustain the release of VEGF for a period of 30 days. The groups treated with VEGF-loaded PLGA nanoparticles demonstrated the maximum healing response when compared to VEGF or PLGA alone (Chereddy et al., 2015). Minimizing the frequency of therapeutics administration is one of the important aspects of an effective therapy. Gainza et al reported that the use of PLGA-alginate microspheres as GFs carriers could significantly minimize the frequency of administration. In their study, single intrallesional injection of PLGA-alginate microspheres carrying a dose of 75μg rhEGF was found to promote complete re-epithelialization in diabetic rats by 11 days (Gainza et al., 2013). Lipid based-particulate systems have also been employed for the delivery of GFs via topical or injection routes. Because of their resemblance with the biological membranes, lipids are particularly attractive for topical application. Cellular internalization of the lipid nanocarriers (solid lipid nanoparticles-SLNs or nanostructured lipid carriers-NLCs) has been demonstrated in vitro.
The significance of this feature was evident on day 8 post-treatment in diabetic mice, where application of two low topical dosage forms of rhEGF loaded SLNs (10 or 20μg) or NLCs (20μg) exhibited greater re-epithelialization than groups treated with two intralesional dosage of free 75μg rhEGF (Gainza et al., 2014).

The studies described above are representative of the various administrative routes applicable with particulate systems. Considering the relative non-invasiveness of the topical approach, identifying the feasibility of using particulate systems for topical GFs delivery as well as an occlusion dressing has become extremely relevant. With large contact surfaces and high bioadhesiveness, and good moisture permeation properties, the particulate systems are logically ideal for topical application. However, the need for frequent topical administration with the particulate systems (Chu et al., 2010) in the attempt to offer optimum therapeutic response may affect patient compliance, for instance, causing frequent disturbance to the wound from unnecessary dressing changes. Therefore, in line to this problem, development of locally implantable therapeutic dressings represents a potential solution. The availability of a wide range of biomaterials and the emergence of advanced fabrication tools and techniques serve as the backbone to finding this solution. The commonly used biomaterials dressings are presented in figure 3. Some notable instances following the application of these dressings are discussed in the following sections. A summary of the corresponding wound healing outcome is also summarized in table 2.
3.2 Modulating the release of GFs from polymeric fibrous mats:

Therapeutic polymeric nanofibrous mats generated using electrospinning is a focus of investigation as wound dressings. These nanofibrous mats intrinsically possess high porosity and surface area which are essential for facilitating the permeation of oxygen and efficient absorption of fluids (Zahedi et al., 2010). Typically, biomolecules are blended into a polymer solution, after which the mixed solution is spun to generate the nanofibers-based delivery systems. Lee et al used this simple approach to develop rhPDGF blended PLGA nanofibers and showed that a prolonged and sustained release of rhPDGF occurred for 21 days and significantly induced complete wound closure in diabetic rats (Lee et al., 2015). Preservation of GF bioactivity against the high proteolytic activity of wound bed is one of the greatest challenges in GF therapy. Choi et al suggested that surface-conjugation of GFs onto the nanofibers is an effective strategy to preserve GF bioactivity following implantation in vivo. They found that implantation of rhEGF conjugated PCL or PCL-PEG nanofibers promoted re-epithelialization through the preservation of keratinocytic phenotype within the wound site. The latter effect was believed to result from strong binding of EGF receptors on keratinocytes with the EGF on nanofibers (Choi et al., 2008). The ability to generate core-sheath fibrous structures using emulsion or co-axial electrospinning has broadened the applicability of nanofibers for GFs delivery. The technique allows encapsulation of GFs within the core-phase of the fibrous construct thereby offering protection of GFs from early proteolysis and also, minimization of the initial burst release. An initial burst release as low as 14.0±2.2% for bFGF encapsulated in the core of an emulsion electrospun poly(ethylene glycol-co-lactide) (PELA) nanofibers has been reported. Synchronously to epithelialization, the fibers were also found to degrade over time (Yang et al., 2011). A co-axially spun core-sheath construct capable of releasing dual-GFs with different release profiles has also been shown to be promising as therapeutic dressing when tested in diabetic mice (Choi et al.,
The construct consisted of bFGF in the core with covalently immobilized EGF on the sheath layer. Early diffusion of bFGF through the thin sheath layer (100-300nm) followed by the release of immobilized EGF via erosion of the sheath matrix were thought to control the release rate (Choi et al., 2011). Tailored release of multiple GFs was reported to induce accelerated wound closure rate, higher collagen deposition and enhanced maturation of vessels than dual-GFs (bFGF and EGF) in vivo. The multiple GFs delivery construct was developed by simultaneous electrospinning of two different precursor solutions, each prepared by dispersing either VEGF-loaded gelatin nanoparticles into bFGF-collagen solution or PDGF-loaded gelatin nanoparticles into EGF-hyaluronic acid solution. When tested in vitro, early and faster release was observed for nanofibers-dispersed GFs (bFGF and EGF) while nanoparticles-bound GFs (VEGF and PDGF) were released in a slow and sustained manner (Lai et al., 2014).

Quite contrary to electrospinning, a group recently reported the use of a layer-by-layer technique to form a hydrolytically degradable tetra-layer architecture consisting of poly(β-amino esters), poly(acrylic acid) (PAA), VEGF and/or PDGF and heparan sulfate over a woven nylon mesh. Electrostatically stacking the GFs between the layers of different polymers allowed the delivery of multiple GFs with distinct release kinetics via surface-based erosion, facilitating a complementary effect of the individual GFs on wound healing (Almquist et al., 2015).

### 3.3 Development of GF loaded 3-dimensional biomaterial dressings:

To date, the terms “sponge” and “foam” have been used interchangeably to represent soft and porous shape-conformable materials. Due to their high absorbency and permeability to moisture and oxygen, both of them are being used as standard wound dressings (Moura et al., 2013). However, the overlapping choice of these dressing materials with those for tissue
engineering applications seems to have covered the disparity between the two forms by mere substitution with the term “scaffold”, which generally represents a temporary platform for guided neo-tissue formation.

By virtue of its abundant occurrence in native ECM, collagen has been an attractive material for the development of biomimetic scaffolds. Collagen based scaffolds alone (Moura et al., 2014) and in combination with other natural polymers such as gelatin (Kanda et al., 2014), hyaluronic acid (Kondo et al., 2012) and chitosan (Wang et al., 2008) or synthetics like poly-glycolic acid (PGA) (Nagato et al., 2006) have been examined for their candidacy as GFs-releasing wound dressings. The freeze-drying technique has been conventionally used to develop such scaffolds (Kondo et al., 2012, Moura et al., 2014, Nagato et al., 2006), which are subjected to crosslinking via chemical (Moura et al., 2014), UV-irradiation (Kondo et al., 2012) or thermal treatment (Nagato et al., 2006) to induce structural stability. From a therapeutic perspective, combining collagen with other polymers to generate a composite scaffold is particularly attractive because it can offer improved resistance to collagenase digestion and also sustain the release of GFs with a slower rate (Wang et al., 2008). Interestingly, one study found that the resistance to degradation of a collagen-gelatin composite scaffold increased correspondingly with higher bFGF loading. However, their in vivo application found that the scaffolds containing 14μg/cm² bFGF induced complete epithelialization and formation of significantly higher density of capillaries than 50μg/cm² groups by 2 weeks. The high dose (50μg/cm²) bFGF delivery was presumed to inhibit keratinocyte proliferation (Kanda et al., 2014).
For local implantation to wounds, incorporating nanoparticles-bound GFs into scaffold is a plausible strategy for two main reasons. First, it would protect the GFs against wound proteases during early course of implantation and second, delay the initial release until induction of healing. A group reported that, with respect to free-GFs loaded scaffolds, application of a fibrin-based scaffold containing VEGF or bFGF loaded PLGA nanoparticles delayed the initial release as well as onset of healing, but demonstrated similar wound closure rate as that of the former by day 15. It is also worth noting that wounds treated with GF loaded scaffolds were found to contain reduced numbers of inflammatory cells as compared to scaffolds without GFs (Losi et al., 2013). Furthermore, maintaining controlled hydration of the wound is essential for stimulating the migration of epidermal cells and epithelialization, and preservation of GFs and cytokines for wound repair (Junker et al., 2013). Hydrophilic polyurethane (PU) formed by co-polymerizing with PEG has been an attractive dressing material for maintaining good moisture conditions in the wound bed. A group had shown that a PU dressing loaded with rhEGF could exhibit a water vapor transmission rate (WVTR) of nearly 3000g/m²/day, which was believed to be desirable for preventing excessive dehydration of the wound. Additionally, the PU dressing was found to sustain the release of rhEGF for up to 7 days in vitro. Implantation of the rhEGF loaded PU dressing promoted re-epithelialization and complete recovery of the wound by 21 days in diabetic rats (Pyun do et al., 2015). Based on a previous report that collagen-binding domain (CBD) linked VEGF (CBD-VEGF) exhibited high affinity for collagen and retainability at granulation tissue (Yan et al., 2010), Tan et al. reverse-applied the concept to develop a collagen scaffold system capable of retaining exogenous VEGF from being washed away by the wound exudates. At 7 days post-implantation, CBD-VEGF loaded collagen scaffold induced significantly higher density of blood vessel formation than native VEGF loaded scaffolds reflecting the high retainability of bioactive concentration of VEGF within the implanted site (Tan et al., 2014).
3.3.1 Hydrogel dressings for controlled release of GFs:

Hydrogels are three-dimensional pre-hydrated dressings. Because of their expandable, highly crosslinked 3-D polymeric network, hydrogels are able to absorb and retain wound exudates, and simultaneously allow water vapor and oxygen transmission to the wound. Hydrogels prepared by 3-D polymerization of monomers are physically irreversible, however due to the generation of significant levels of toxic residual monomers during the polymerization and the likeliness of their leakage from the prepared hydrogels, chemical modifications aimed at developing crosslinking-ready water-soluble polymers gained broader acceptance. Adoption of the latter avoided hydrogel purification and allowed utilization of rapid and more inexpensive crosslinking-sterilization techniques such as UV-irradiation (Caló and Khutoryanskiy, 2015). Exposure of FGF-2 containing photo-crosslinkable chitosan solution to UV reportedly induced the formation of insoluble and flexible hydrogel within 30 seconds and its subsequent application facilitated wound closure with complete epithelialization by 16 days in diabetic mice (Obara et al., 2003). In another instance, the high-binding affinity of bFGF to heparin in the native ECM influenced the development of biomimetic hydrogel films composed of crosslinkable derivatives of chondroitin-6-sulfate and heparin for the controlled delivery of bFGF into subcutaneous wounds of genetically diabetic mice. The presence of heparin was speculated to have acted synergistically in modulating the release of bFGF, and allowed optimal healing with low (2μg) or intermediate (10μg) dose delivery of bFGF (Liu et al., 2007). Furthermore, a study showed that selective de-sulfation of heparin could modulate the immobilization efficiency and release of VEGF-A from StarPEG-heparin composite hydrogels, implying an attractive avenue for the development of dose-adjustable GFs delivery system containing sulfated glucosaminoglycans (GAGs). With increasing de-sulfation, immobilization efficiency of VEGF-A was decreased while the initial release kinetics was inversely affected. Implantation of the hydrogel with 0.1 μg or 1 μg VEGF-A per wound
revealed significant granulation tissue and neo-epithelium formation at 10 days post-wounding in diabetic mice (Freudenberg et al., 2015). Alternatively, integration of GF-conjugation strategies has been found to improve the therapeutic approaches utilizing gels for topical application. One study showed that topical application of dextrin-rhEGF conjugate formulated at concentrations equivalent to the presence of rhEGF at 1 or 10 μg/ml significantly promoted wound closure in diabetic mice as compared to free rhEGF at 10 μg/ml (Hardwicke et al., 2011). Hydrogels (Hajimiri et al., 2015) and gels (Yeboah et al., 2016) were also employed to deliver GF-conjugate nanoparticles into diabetic wounds. The nanoparticles employed in the studies were formed by either chemically conjugating the GFs with polymers (e.g. rhEGF-sodium carboxymethyl chitosan (Hajimiri et al., 2015)) or by recombinantly fusing them (e.g KGF (Koria et al., 2011) or SDF-1 (Yeboah et al., 2016)) with elastin-like peptides (ELP). Clearly, the emphasis of these studies was with the development of these therapeutic nanoparticles, that offered improved resistance to degradation by protease (savinase (Hajimiri et al., 2015) and elastase (Yeboah et al., 2016)) thereby enhancing the therapeutic effect in vivo.

4. Gene-mediated therapeutic delivery:

In principle, gene-mediated therapeutic delivery involves the localized transfection of therapeutic transgene or complementary deoxyribonucleic acid (cDNA) into the cells, which then gets transcribed into messenger ribonucleic acid (mRNA) and their translation into the encoded protein in situ (Raftery et al., 2016). Initially, the concept of gene therapy was applied to permanent correction of genetic disorders, whose curative effect was assessed by long-term transgene expression. Conversely, transient gene therapy is of particular interest for local disorders since a transient increase in strategic transgene expression is required until complete tissue repair is achieved (Branski et al., 2009). The concept has been put forward for therapeutic investigations in degenerative disorders as it may possibly minimise systemic
effects by sustaining high GF concentrations at the targeted site, increase the production of more precisely modified biologically active structure with more recognizable ligands than recombinant proteins. Also, the relatively stable nature with long shelf life of cDNA, ease of large scale production and low-cost favours the rationale for gene delivery (Southwood et al., 2004). To this end, modulating the degree of gene expression using non-integrating expression vectors that could avoid undesirable effects arising from host genome integration remains the holy-grail of regenerative gene medicine (Colosimo A, 2000). Typical design strategy is aimed at equipping such vectors for localizing and maintaining extrachromosomally within the host’s cell (Jackson et al., 2006). This section will cover the trends in the development of non-integrating expression vectors and assessment of their transfection efficiency for transient gene therapy in diabetic wound healing. Of particular relevance to wound healing, skin is an amenable organ for genetic manipulations and renders an easy in vivo approach as well as follow-up of therapeutic effects. The high turnover of the epidermis and the fact that a multitude of cytokines and GFs crucial to the regeneration process undergo short-term up- and down-regulation make it an ideal target tissue for gene therapy (Bleiziffer et al., 2007).

4.1. Delivering plasmid DNAs in diabetic wounds:

Naked plasmids represent the simplest form of non-integrating expression vector. It has a very large DNA packaging capacity and can accommodate large segments of genomic DNA. Regrettably, the large size (1-200kbp) and the presence of phosphodiester backbone offers an overall anionic charge making it difficult for cellular internalization (Raftery et al., 2016). Furthermore, susceptibility of plasmid DNA (pDNA) to rapid clearance by system macrophages and nuclease degradation contributes largely to its low transfection. Therefore, delivery of pDNA into the tissues or cells are assisted with the application of physical methods such as electroporation, sonoporation, hydroporation, laser irradiation, particle
bombardment (gene gun) or magnetofection, whose principles are based on improving the kinetics of plasmid transfer via temporal permealization of cell membrane upon application (Mehier-Humbert and Guy, 2005, Wells, 2004). Early studies on diabetic wounds focussed on forcing the pDNAs into the cells by intradermal injections (Byrnes et al., 2001, Sophie Chesnoy, 2003), which later performed in conjunction or adjuvant with electroporation, higher pDNA uptake was achievable (Lee et al., 2004, Liu et al., 2008, Marti et al., 2004). The relative ease and straightforwardness in applicability, non-invasiveness, ability to target large cell populations and stimulate epithelial cells proliferation (Lee et al., 2004) have perhaps favoured greater utilization of electroporation as a physical method for pDNA delivery into diabetic wounds. Nevertheless, these techniques require constant optimization of physical parameters (field strength, field distribution and exposure time) depending upon the sensitivity of the tissue. For instance, in diabetic skin, electroporation with low applied voltage of 100V/cm with 20 ms pulse interval was optimum to avoid tissue damage and at the same time achieve 10 fold higher transfection than that of normal skin under the same conditions (Lee et al., 2004). Recently, ultrasound micro-bubbling agents (SonoVue™) assisted sonoporation was used to enhance the delivery of VEGF_{165} encoded minicircles (small modified plasmids) into the wounds of diabetic mice. Application of the ultrasound at a frequency of 1.0 MHz with an exposure intensity and duty cycle at 2.0 W/cm² and 20% respectively, for 30 seconds was sufficient to maintain higher transfection than pDNAs alone, consequently higher perfusion and wound closure percentage in diabetic mice (Yoon et al., 2009). Principally, the increase in transfection with these methods could be related to higher cytosolic transport of pDNAs, facilitating greater pDNA accumulation in the vicinity of the nucleus. In general, it might serve as a fundamental that applications of any mechanical stimulus be performed with minimized levels of operating parameters as diabetic skins are characterized with reduced dermal thickness and hence, highly susceptible to damage
This feature of diabetic skin could also account for the deteriorating utilization of physical methods for pDNA delivery. To further substantiate nuclear transduction, attention has been drawn to the development of carriers that can either deliver the genes directly (viral vectors) or the plasmids encoding the gene of interest (chemical vectors).

### 4.2 Transfection of therapeutic genes with non-integrating viral vectors:

The intrinsic ability of viruses to integrate into host genome and advancements in the design of integration- and replication-defective viruses offer significant advantages in the delivery of therapeutic genes to mammalian cells. Notably, viruses capable of efficiently transfecting both dividing and non-dividing cells have been the most explored for diabetic wound healing. These viruses include adenovirus (AV), lentivirus (LV) and adeno-associated virus (AAV). The AV due to its large genome size packaging capacity (up to 30kbp) and more importantly, the non-integrating nature (Boeckle S, 2006, Zhang and Godbey, 2006) makes it very desirable for use as vector that allows transient expression of the therapeutic gene. One of the early studies reported that transfection of VEGF$^{165}$ gene with a replication-deficient AV induced earlier wound healing response in diabetic mice leading to an apparent difference in wound closure at day 3 when compared to either untransfected diabetic (control) or non-diabetic mice. Additionally, the transfection of VEGF gene promoted angiogenesis and granulation tissue formation thereby facilitating the recovery of wound at a similar rate as that of non-diabetic mice (Romano Di Peppe S, 2002). Later in 2009, it was reported that AV mediated VEGF delivery increased keratinocyte migration and collagen deposition, contributing to re-epithelialization and thicker granulation tissue formation, respectively. The collagen fibers within the neo-granulation tissue were found to be long and arranged in an organized manner, the credit for which, was attributed to the presence of AV (Brem et al., 2009). Similar formation of aligned collagen bundles were also observed upon transfecting
PDGF gene with LV (Lee et al., 2005) but surprisingly, PDGF gene transferred adenovirally was found to influence random deposition of short collagen fibers (Keswani et al., 2004). Of note, deposition of aligned collagen fibers is a prerequisite for accelerated formation of mature granulation tissue.

The rapid transfection with viral vectors and early induction of healing response suggest that transfection at early injury phase can rescue the wound from progression to its chronicity. For instance, immediate exposure of stromal-derived factor-1 alpha (SDF-1 α; a chemokine) encoded LV post-wounding, yielded early development of granulation tissue with high dermal cellularity and avoided progression to persistent inflammatory phase in diabetic mice. In vitro transfection of dermal fibroblasts by the LV yielded a transfection efficiency of 95% in 3 days (Badillo et al., 2007). Although it is indicated that the modified non-integrating LV or AAV may also be suitable for application in wounds desiring longer therapeutic effect of the transgene (Shaw and Cornetta, 2014, Zhang and Godbey, 2006), modulating the expression of transgene for a therapeutically significant period of time has remained a challenging task. Such circumstance was reported by a group where they found that even after the recovery of normalized skin (28 days), VEGF transgene expression mediated via AAVs persisted for up to 4 months in vivo (Galeano et al., 2003).

Irrespective of the type of vector or duration of transgene expression, transfection of either a single GF (Botusan et al., 2008) or different GF-isoform (Saaristo et al., 2006) genes activated only the corresponding signaling pathways, which may not be sufficient to promote the multiple phases of wound healing (Gauglitz and Jeschke, 2011). In light of this problem, the use of a single viral vector capable of transfecting multiple genes (polycistronic viruses) may be advantageous. Design strategies for such viruses have been discussed in detail elsewhere (Felipe, 2002). Sustained transgene expression for a period of 21 days and the
superiority over single GF (FGF4) delivery in diabetic wound healing was demonstrated with the simultaneous delivery of VEGF-A and FGF4 genes via bicistronic AAVs (Jazwa et al., 2010). All the above studies suggested that the transgene expression was confined to the skin, which is a clinically important setting for avoiding systemic effects to the nearby tissues such as muscle. However, their unpredictable nature such as continual expression beyond a desirable timeline (Galeano et al., 2003) and the risk of causing insertional mutagenesis that occurs as a result of activation of cell-growth regulatory genes within the virus (Shaw and Cornetta, 2014) remain as major limitations with virus based-gene delivery. Inconsistency with reproducibility of high titers and purity further limits their progression in applications for gene delivery. To date, refining the safety of viral vectors is maintained as top-priority for therapeutic translation (Sinn et al., 2005). The various delivery routes adopted for the delivery of viral vectors are presented in Table 1.

4.3 Therapeutic gene delivery using chemical vectors:

The emerging interest in the use of chemical vectors such as the cationic polymers and lipids results from their ability to form electrostatic complexes with anionic biomolecules such as the pDNA (Lv et al., 2006). The adoption of these chemical vectors could not only avoid the use of potentially immunogenic viruses but also improve the biostability of pDNAs, facilitate cellular uptake and undergo endolysosomal escape (Samal et al., 2012). The transfection systems resulting from the use of cationic polymer or lipids are termed polyplex or lipoplex respectively. The transfection efficiency as well as cytotoxicity for such systems is closely dependent upon the charge ratio between the cationic and anionic species in the polymer/lipid and DNA respectively (Lv et al., 2006). Commonly used cationic polymers for gene delivery include polyethyleneimine (PEI) and poly-l-lysine (PLL) which are water-soluble. However, the lipids, because of their amphiphilic nature has gained considerable interest for topical
applicability as it can facilitate easy permeation through the multiple hydrophobic-hydrophilic domains in the skin (Geusens et al., 2011). A study was reported as early as 1997, where combined topical and subcutaneous administration of acidic FGF cDNA with cationic liposomes on a daily basis resulted in increased wound strength and accelerated wound closure in diabetic mice (Sun et al., 1997). Studies in the recent years demonstrated that the versatility for modifications with chemical vectors offers the advantage to translate into more effective carrier for gene delivery that can allow reduction in administration frequency as well as dosage. For instance, single dose subcutaneous injection of lipoplex formed by complexing integrin receptor ligand (RGDK) conjugated lipopetide with rhPDGF-B (50μg pDNA) (Bhattacharyya J, 2009) or polyplex composed of positively charged arginine grafted dendrimer (Starbust) and minicircle VEGF (20μg pDNA) (Kwon et al., 2012), was able to induce complete wound closure in diabetic mice by 12 days. The induction of healing with lipoplex was attributed to the high selectivity of the ligand for proangiogenic α5β1 receptors on fibroblasts (Bhattacharyya J, 2009) while with polyplex, it was due to the transfection of rapidly proliferating basal cells (Kwon et al., 2012). Surprisingly, despite these evidences for success, a lack of consistent in vivo gene delivery studies using chemical vectors is notable.

4.4 Gene-eluting biomaterial constructs:

Development of biomaterial constructs capable of delivering therapeutic genes, as an approach for regenerative therapy is still in its early phase and its application in vivo, particularly in diabetic wound healing has been limited to date. The process of developing gene-eluting biomaterial scaffolds shares similar design principles as that of GF-loaded scaffolds but with an emphasis directed to improving vector stability and promoting and/or controlling cell-vector interactions to modulate the location and duration of transgene expression (Seidlits et al., 2013).
In one of the early attempts, Lee and coworkers developed a purely synthetic (PEG-PLGA-PEG) thermosensitive hydrogel capable of in situ gelation upon local application to the wound and also controlling the release of encapsulated plasmid (TGF-β1). The application of the hydrogel elicited significant acceleration of re-epithelialization at early healing stages (day 1-5) (Lee et al., 2003). This study is in particular, representative of the customizable nature of hydrogels (stimuli sensitive) for spanning the gap between conventional gel-based systems and solid scaffolds for gene delivery to open wounds. More recent studies have utilised polyplexes, with a focus on modulating their release for efficient gene transfection. In a case using VEGF/PEI polyplex loaded hyaluronic acid hydrogel, the porosity of the hydrogel was considered a fundamental parameter for rapid cellular infiltration, and provide large contact area for maximum encounter of the released polyplexes with the infiltrating cells. However, due to the electrostatic interaction between positively charged polyplexes and anionic hyaluronic acid matrix, the release of polyplex was believed to be very slow to yield high levels of transfected infiltrated cells. Hence the contribution on wound closure as a result of angiogenesis by transfected cells was considered to be insignificant (Tokatlian et al., 2015). Nevertheless, the fact that the positively charged polyplex interacts electrostatically within the anionic hyaluronic acid hydrogel matrix serves as a basis for the development of more controllable polyplex delivery systems. Yang et al. showed that the release of polyplex (plasmid bFGF/PEI) encapsulated within the core of core-sheath emulsion electrospun PELA fibers could be modulated by changing the molecular weights and contents of PEG within the copolymer matrix. Interestingly, their in vitro release and transfection period (28 days) was in accordance with the duration of wound recovery in vivo (Yang et al., 2012). Surface conjugation of fixed amount of linear PEIs (LPEI) onto nanofibers via MMP-responsive linkers offers another strategic approach for developing a polyplex releasing system. Kim et al. found that this strategy allowed tunability in the amount of pDNA to be incorporated in
the LPEI-immobilized nanofibers and their release as polyplexes following enzymatic cleavage of the linkers resulting in cellular transfection (Kim and Yoo, 2013).

In diabetic wounds, issues concerning the low cell availability at the wound edges and their diminished migration (Brem and Tomic-Canic, 2007, Lerman et al., 2003, Xuan et al., 2014) at the wound site may add to the difficulties in modulating the transfection efficiency in vivo with gene-eluting biomaterial systems. Furthermore, the condition of constant glycation activities in diabetes could present an additional barrier to delivering the desired therapeutic effect. A recent study by Thiersch et al. found that release of nanocondensates formed by combining PLL-grafted-PEG and HIF-1α plasmid from fibrin hydrogel significantly induced HIF-1 downstream target VEGF gene expression in healthy animals, but failed to imitate the effect in diabetic animals. The authors implicated that the methylglyoxylation of its transcriptional cofactor p300 inhibited the corresponding downstream signaling cascades (Thiersch et al., 2013).

To date, the only clinical trial performed with such gene-eluting biomaterial systems has been with PDGF-B encoded replication-deficient AV formulated within a collagen gel, also termed gene-activated matrix 501 (GAM501). Patients with ulcer area 1-10 cm² were recruited for the study. Assessment of clinical safety and efficacy conducted in phases 1A and 1B trial found that, a single topical application of GAM501 at 1x10¹⁰, 3x10¹⁰, or 1 x10¹¹ v.p/cm² (v.p - viral particles) wound bed exhibited similar biologic response as that of multiple applications (up to 4 topical applications) at single dose level of 3x10¹⁰ v.p/cm² at 1-week intervals (Mulder et al., 2009). A year later, clinical comparison between GAM501, formulated collagen gel (FCG) and standard of care (SOC) identified that single application of either GAM501 or FCG had a significant healing effect within the first 2 weeks than multiple weekly SOC interventions (Blume et al., 2011). Figure 4 represents enhanced
healing of foot ulcers following the treatment with GAM501 in phase 1/2 trial.

5. State of the art and future reviews:

Having recognized the difficulty imposed upon by wound environment on the bioactivity of localized therapeutics, adoption of an interdisciplinary approach for improving the therapeutics delivery is highly anticipated as a promising solution. As such, our review aimed at providing researchers with information on the various effects corresponding to their rationale on the choice of GF (or genes) or combination of GFs followed by strategic optimization of delivery strategies adopted (injection or topical) for healing of diabetic wounds. Gene delivery has the advantage that it can sustain the production of protein of interest in situ, however the lack of clinically approved chemical vectors and the concerns associated with potentially immunogenic viruses still maintains as major limitations. Additionally, the choice of administrative route could have a significant effect on patient compliance. As it could be evident from tables 1 and 2 that, while gene delivery has been predominantly performed via injection of viral particles encoding for the therapeutic gene, most biomaterial systems have been applied topically which is relatively non-invasive than the former approach. The application of implantable biomaterials not only favours the sustained release of localized concentration of bioactive GFs in the wound site but also offers the advantage of acting as an occlusive without necessitating frequent renewals following implantation. Gene-eluting biomaterial systems also holds potential for the repair of diabetic wounds although modulating the cell-vector interaction remains critical for succesful induction of the therapeutic effect. In particular relevance to diabetic wound healing, in vitro studies relating to gene transfer or angiogenic effect of a particular GF should be performed with cells harvested from diabetic patients, and the succeeding result could be applied for development of more advanced tissue-engineered wound dressings. Since diabetes is a
metabolic disorder, additional strategies targeted to normalizing blood glucose level should also be considered for exerting maximum effect of the delivered therapeutics.

Acknowledgement:

This study was supported by the Royal College of Surgeons in Ireland Dilmun PhD scholarship.

Conflict of interest:

The authors declare that no conflict of interest exists.

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Figure 1. A schematic depicting normal wound healing process. Abbreviation:

TIMPs—tissue inhibitors of metalloproteinases
Figure 2. Hyperglycemia impairs angiogenesis and wound healing.
Figure 3. The different formats of commonly adopted therapeutic dressings. (I) Electrospun nanofibers 1. Direct blending of GFs 2. Encapsulation of GFs in the core of a core-shell construct 3. Incorporation of nanoparticles bound GFs 4. GFs conjugated on the surface of fibers 5. Encapsulation of GFs in the core of a core-shell followed by surface-conjugation of another GF; (II) Hydrogels and 3-D scaffolds a) Entrapment of GFs within the hydrogel matrix b) Entrapment of GFs within the porous scaffold c) Micro/Nanoparticles bound GFs embedded into hydrogel d) GFs chemically conjugated onto scaffolds e) Incorporating micro/nanoparticles bound GFs into scaffold.
Figure 4. Representative images of healing of foot ulcers following the treatment with GAM501 in phase 1/2 clinical trial (Mulder et al., 2009).
Table 1. Gene delivery vectors applied for the treatment of diabetic wounds

<table>
<thead>
<tr>
<th>Mode of administration</th>
<th>Therapeutics carrier (Applied doses)</th>
<th>Diabetic animal model</th>
<th>Wound geometry</th>
<th>Response on wound closure</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intradermal injection</td>
<td>Plasmid KGF-1 (100μg)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 5 mm diameter</td>
<td>Enhanced wound closure at day 9</td>
<td>Byrnes, 2001</td>
</tr>
<tr>
<td>Intradermal injection</td>
<td>Plasmid TGF -β1 (60μg)</td>
<td>Genetically diabetic mice</td>
<td>Square, 1 cm x 1 cm</td>
<td>Complete wound closure by 7 days</td>
<td>Shophie, 2003</td>
</tr>
<tr>
<td>Intradermal injection; Electroporation</td>
<td>Plasmid TGF -β1 (30μg)</td>
<td>Genetically diabetic mice</td>
<td>Square, 7 mm x 7 mm</td>
<td>Early induction of closure by day 5</td>
<td>Lee, 2004</td>
</tr>
<tr>
<td>Intradermal injection; Electroporation</td>
<td>Plasmid KGF-1 (100μg)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 5 mm diameter</td>
<td>Enhanced wound closure at day 12</td>
<td>Marti, 2004</td>
</tr>
<tr>
<td>Intradermal injection; Electroporation</td>
<td>Plasmid HIF-1α (50μg)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 5 mm diameter</td>
<td>60% wound closure at 10 days</td>
<td>Liu, 2008</td>
</tr>
<tr>
<td>Subcutaneous injection; Sonoporation</td>
<td>Minicircle-VEGF (20μg)</td>
<td>STZ-diabetic mice</td>
<td>Circular, 6 mm diameter</td>
<td>Complete wound closure by 12 days</td>
<td>Yoon, 2009</td>
</tr>
<tr>
<td>Topical application to wound bed</td>
<td>Adenovirus encoding VEGF (10^8 p.f.u)</td>
<td>STZ-diabetic mice</td>
<td>Circular, 3.5 mm diameter</td>
<td>Complete wound closure by 13 days</td>
<td>Romano, 2002</td>
</tr>
<tr>
<td>Intradermal injection</td>
<td>Adenovirus encoding VEGF (5 x 10^{10}, 5 x 10^{11}, 1.6 x 10^{10}, 1.6 x 10^{11} v.p)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 1.4 cm diameter</td>
<td>Complete wound closure by 27 days</td>
<td>Brem, 2009</td>
</tr>
<tr>
<td>Intralional injection</td>
<td>Adenovirus encoding PDGF (10^8 p.f.u)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 8 mm diameter</td>
<td>Residual epithelial gap of 3 mm at day 7</td>
<td>Keswani, 2004</td>
</tr>
<tr>
<td>Intradermal injection</td>
<td>Adenovirus encoding VEGF-C (5 x 10^8 p.f.u)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 3-5 mm diameter</td>
<td>Complete wound closure by 21 days</td>
<td>Saaristo, 2006</td>
</tr>
<tr>
<td>Intradermal injection</td>
<td>Adenovirus encoding HIF (10^8 p.f.u)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 6 mm diameter</td>
<td>Not defined</td>
<td>Botusan, 2008</td>
</tr>
<tr>
<td>Injected into base and wound margin</td>
<td>Lentivirus encoding PDGF (10^6 transducing units)</td>
<td>Genetically diabetic mice</td>
<td>Square, 2 cm x 2 cm</td>
<td>No statistically significant residual epithelial gap as compared to untreated groups at day 21</td>
<td>Lee, 2005</td>
</tr>
<tr>
<td>Intradermal injection</td>
<td>Lentivirus encoding SDF-1α (10^8 p.f.u)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 8 mm diameter</td>
<td>Enhanced wound closure at day 7</td>
<td>Badillo, 2007</td>
</tr>
<tr>
<td>Intradermal injection</td>
<td>Adeno-associated virus encoding VEGF (10^{11} v.p)</td>
<td>Genetically diabetic mice</td>
<td>Not defined</td>
<td>Complete re-epithelialization at 28 days</td>
<td>Galeano, 2003</td>
</tr>
<tr>
<td>Intradermal injection</td>
<td>Bicistronic Adeno-associated virus encoding VEGF-A and FGF4 (3 x 10^{10} v.p)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 4 mm diameter</td>
<td>Complete wound closure by 17 days</td>
<td>Jazwa, 2010</td>
</tr>
<tr>
<td>Subcutaneous injection</td>
<td>RGDK-lipopeptide:rhPDGF-B lipoplex (50μg pDNA)</td>
<td>STZ-diabetic rats</td>
<td>Circular, 2.1 cm radius</td>
<td>Complete wound closure by 12 days</td>
<td>Bhattacharyya, 2009</td>
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<tr>
<td>Subcutaneous injection</td>
<td>Minicircle VEGF:Cationic dendrimer polyplex (20μg pDNA)</td>
<td>STZ-diabetic mice</td>
<td>Not defined</td>
<td>Complete wound closure by 12 days</td>
<td>Kwon et al, 2012</td>
</tr>
</tbody>
</table>

Abbreviations: p.f.u – particle forming units; v.p – viral particles
Table 2. Biomaterial systems applied for the delivery of growth factors and genes in diabetic wounds

<table>
<thead>
<tr>
<th>Particulate systems for the sustained release of growth factors into diabetic wounds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Delivery route</strong></td>
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<tr>
<td>---------------------</td>
</tr>
<tr>
<td>Topical spraying to wound bed</td>
</tr>
<tr>
<td>Intradermal injection</td>
</tr>
<tr>
<td>Intralesional injection</td>
</tr>
<tr>
<td>Topical application to wound bed</td>
</tr>
</tbody>
</table>

**Locally implantable biomaterial systems for growth factors delivery to diabetic wound bed**

<table>
<thead>
<tr>
<th><strong>Type of implant</strong></th>
<th><strong>Type of formulation (loading amount)</strong></th>
<th><strong>Diabetic animal model</strong></th>
<th><strong>Wound geometry</strong></th>
<th><strong>Response on wound closure</strong></th>
<th><strong>Reference</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-woven mesh (Electrospun)</td>
<td>rhPDGF/PLGA (0.005g/0.28g)</td>
<td>STZ-diabetic rats</td>
<td>Circular, 8 mm diameter</td>
<td>Complete wound closure by 14 days</td>
<td>Lee at al, 2015</td>
</tr>
<tr>
<td></td>
<td>rhEGF/PCL, PCL-PEG (0.00072nmol/mg)</td>
<td>STZ-diabetic mice</td>
<td>Circular, 8 mm diameter</td>
<td>Accelerated wound closure at 7 days</td>
<td>Choi et al, 2008</td>
</tr>
<tr>
<td></td>
<td>bFGF/PELA (0.079±0.015% w/w)</td>
<td>STZ-diabetic rats</td>
<td>Circular, 250 mm²</td>
<td>Complete wound closure by 3 weeks</td>
<td>Yang et al, 2011</td>
</tr>
<tr>
<td></td>
<td>bFGF, rhEGF/PCL, PCL-PEG (0.98μg of bFGF and 9.4μg of rhEGF per device)</td>
<td>STZ-diabetic rats</td>
<td>Circular, 8 mm diameter</td>
<td>Accelerated wound closure at 7 days</td>
<td>Choi et al, 2011</td>
</tr>
<tr>
<td></td>
<td>VEGF, bFGF, EGF, PDGF/Collagen, Hyaluronic acid, Gelatin nps (5μl of VEGF or PDGF at 0.2μg/ml per 10 mg of gelatin nps)</td>
<td>STZ-diabetic rats</td>
<td>Circular, 15 mm diameter</td>
<td>Complete wound closure by 4 weeks</td>
<td>Lai et al, 2014</td>
</tr>
<tr>
<td>Woven nylon</td>
<td>VEGF,</td>
<td>Genetically</td>
<td>Circular, 6 mm</td>
<td>Accelerated</td>
<td>Almquist et</td>
</tr>
<tr>
<td>mesh</td>
<td>PDGF/Poly(β-amino esters), PAA, Heparan sulfate (40 tetralayer repeats of PDGF or VEGF per section)</td>
<td>diabetic mice</td>
<td>diameter</td>
<td>wound closure at 14 days</td>
<td>al, 2015</td>
</tr>
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<tr>
<td>bFGF/Collagen, Gelatin (7-14μg/cm²)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 8 mm diameter</td>
<td>Not defined</td>
<td>Kanda et al, 2014</td>
<td></td>
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<tr>
<td>EGF/Collagen, Hyaluronic acid (2μg/cm²)</td>
<td>Genetically diabetic mice</td>
<td>1.5 cm x 2 cm</td>
<td>Not defined</td>
<td>Kondo et al, 2012</td>
<td></td>
</tr>
<tr>
<td>aFGF/Collagen, Chitosan (4μg/2.5cm³)</td>
<td>STZ- diabetic rats</td>
<td>Circular, 1.8 cm diameter</td>
<td>Complete healing at 14 days</td>
<td>Wang et al, 2008</td>
<td></td>
</tr>
<tr>
<td>bFGF/Collagen, PGA (100μl at 100μg/ml)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 6 mm diameter</td>
<td>Not defined</td>
<td>Nagato et al, 2006</td>
<td></td>
</tr>
<tr>
<td>VEGF/bFGF loaded PLGA nps, Fibrin (20mg of nps containing 200ng of VEGF or bFGF)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 8 mm diameter</td>
<td>85% wound closure at 15 days</td>
<td>Losi et al, 2013</td>
<td></td>
</tr>
<tr>
<td>rhEGF/PU (8.4μg/cm²)</td>
<td>STZ- diabetic rats</td>
<td>Square, 2 cm x 2 cm</td>
<td>97% wound closure at 21 days</td>
<td>Pyun do et al, 2015</td>
<td></td>
</tr>
<tr>
<td>FGF-2/Chitosan (250ng/50μl)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 100 mm²</td>
<td>80% wound closure by 12 days</td>
<td>Obara et al, 2003</td>
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</tr>
<tr>
<td>bFGF/Chondroitin -6-sulfate, Heparin (20μg/film)</td>
<td>Genetically diabetic mice</td>
<td>Circular, 1.6 cm diameter</td>
<td>89% wound closure by 2 weeks (20ug)</td>
<td>Liu et al, 2007</td>
<td></td>
</tr>
<tr>
<td>VEGF/PEG, Heparin (0.1 or 1μg/gel)</td>
<td>Genetically diabetic mice</td>
<td>Not defined</td>
<td>Not defined</td>
<td>Freudenberger et al, 2015</td>
<td></td>
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<tr>
<td>rhEGF-Sodium carboxymethyl chitosan nps/ PVP (0.25 ml of nps solution/2.5 ml of PVP)</td>
<td>STZ-diabetic rats</td>
<td>Circular, 2 cm diameter</td>
<td>Negligible wound area at day 15</td>
<td>Hajimiri et al, 2015</td>
<td></td>
</tr>
</tbody>
</table>

**Gene-eluting biomaterial systems applied for the treatment of diabetic wounds**

<table>
<thead>
<tr>
<th>Hydrogels</th>
<th>pDNA TGF-β1/PEG-PLGA-PEG (200μg/70μl)</th>
<th>Genetically diabetic mice</th>
<th>Square, 7 mm x 7 mm</th>
<th>Accelerated wound closure at 5 days</th>
<th>Lee et al, 2003</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogels</td>
<td>pDNA/VEGF-PEI polyplex/Hyaluron</td>
<td>Genetically diabetic mice</td>
<td>Circular, 6 mm diameter</td>
<td>Induction of wound closure</td>
<td>Tokatlian et al, 2015</td>
</tr>
<tr>
<td>Method</td>
<td>Treatment</td>
<td>Model</td>
<td>Outcome</td>
<td>Reference</td>
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<tr>
<td>Electrospun mesh</td>
<td>pDNA bFGF-PEI polyplex/PELA (50μl of polyplex solution at 1mg/ml pDNA per 450mg PELA)</td>
<td>STZ-diabetic rats</td>
<td>Complete wound closure by 3 weeks</td>
<td>Yang et al, 2012</td>
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<td></td>
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<td>Circular, 250 mm²</td>
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<td></td>
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<td>STZ-diabetic mice</td>
<td>Enhanced wound closure at day 7</td>
<td>Kim et al, 2013</td>
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<td>Circular, 9 mm diameter</td>
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<tr>
<td>Epidermal acid (lyophilized polylex containing 250μg pDNA per 100μl gel)</td>
<td></td>
<td>by days 8-10</td>
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<tr>
<td>pDNA HIF-PLL grafted PEG nanocondensates/Fibrin (nanocondensates carrying 1μg pDNA per 100μl gel)</td>
<td>STZ-diabetic rats</td>
<td>Circular, 10 mm diameter</td>
<td>Not defined</td>
<td>Thiersch et al, 2013</td>
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<tr>
<td>pEGF-linear PEI/PCL-PEG (6.8μg/device)</td>
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