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Changing the Wound: Covalent Immobilization of the Epidermal Growth Factor

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⊗Author Contributions: V.R., S.A.P., N.M.S. contributed equally to this work. V.R., S.A.P., N.M.S., R.R.L., N.L.A., and C.J.M. were involved in the study design. V.R., S.A.P., N.M.S., C.M.R., L.T., R.D., Y.-R.C., and M.J.M. were involved in conducting experiments and data analysis. M.J.S. and J.F.M. helped interpret animal studies and provide clinical context. V.R., S.A.P., N.L.A., and C.J.M. wrote the manuscript. All authors edited and approved manuscript.

The authors declare the following competing financial interest(s): NLA, JM, MS and CJM are co-founders of Imbed Biosciences Inc., an advanced wound care company that markets biomaterials with antimicrobial properties.

All data are presented in the manuscript and Supporting Information Raw data are available if requested.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomaterials.1c00192.
Histopathological evaluations with/without topically applied EGF; in vitro proliferation rates of spontaneously immortalized human keratinocytes; validation of use of the heterobifunctional crosslinker using Gamma-Graft; HaCaT and HMVEC viability affected by sulfo-SMCC or TCEP; and percentage distribution of inflammatory cells in the wound bed (PDF)

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Abstract

Re-epithelialization of wounds is a critical element of wound closure. Growth factors have been used in combination with conventional wound management to promote closure, but the method of delivery has been limited to the topical application of ointment formulations. Cytoactive factors delivered in this way have short resident times in wounds and have met with limited success. Here, we demonstrate that methods used to covalently immobilize proteins on synthetic materials can be extended to immobilize cytoactive factors such as the epidermal growth factor (EGF) onto the wound beds of genetically diabetic mice that exhibit impaired healing. Full-thickness splinted excisional wounds were created in diabetic (db/db) mice with a well-defined silicone splint to limit wound contracture. Wound surfaces were treated with a reducing agent to expose sulfhydryl groups and subsequently treated with EGF modified with a heterobifunctional crosslinker. This allowed for the covalent immobilization of the EGF to the wound surface. The conjugation chemistry was validated in vitro and in vivo. In a separate group of mice, wounds were topically treated twice daily with soluble EGF. The mice were evaluated over 11 days for wound closure. This covalent immobilization strategy resulted in EGF being retained on the wound surface for 2 days and significantly increased epithelial wound closure by 20% compared to wounds treated

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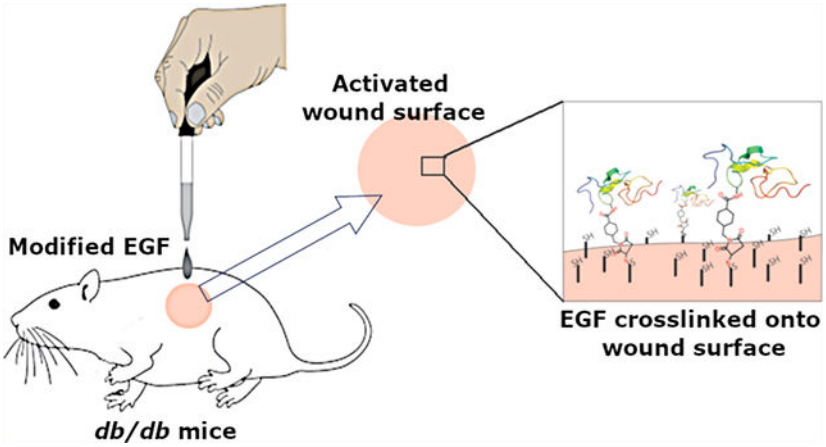
with topical EGF or topical vehicle. Covalent immobilization was not only therapeutically effective but also delivered a markedly reduced load of growth factor to the wound surface compared to topical application (when only 180 ng of EGF was immobilized onto the wound surface in comparison with 7200 ng of topically applied EGF over a period of 11 days). No adverse effects were observed in treated wounds. Results obtained provide proof of concept for the effectiveness of covalent immobilization in the treatment of dysregulated wounds. The covalent immobilization of cytoactive factors represents a potentially transformative approach to the management of difficult chronic wounds.

Graphical Abstract



Keywords

covalent immobilization; bioconjugation; wound healing; epidermal growth factor; diabetes

INTRODUCTION

Chronic wounds encompass a variety of conditions such as venous leg ulcers, pressure ulcers, vascular insufficiency, and diabetic foot ulcers to name a few. They are a significant burden to patients with profound effects on their quality of life and on healthcare costs worldwide. In 2009, in the United States, chronic wounds were reported to affect around 6.5 million patients with an excess of $25 billion spent annually on their treatment.[1,2] More recently, the American Diabetes Association, in 2013, estimated that the total economic burden for “diagnosed diabetes” in the US alone was a staggering $245 billion attributed to the increasing cost of care with rising prevalence, demographics, and changes in treatment access, modalities, and comorbidities.[3,4] These costs are only expected to rise and suggest that cost-effective treatment and care would significantly alleviate future economic burden.

Wound healing is a complex process with highly integrated and overlapping phases of hemostasis, inflammation, epithelialization, formation of granulation tissue, neovascularization, wound contraction, and extracellular matrix (ECM) reorganization (Figure 1). A critical clinical endpoint considered by the US Food and Drug Administration for wound closure is re-epithelialization, [5–8] a process that can be prolonged in pathologic

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wounds (e.g., diabetes, venous stasis, and denervation) as well as extensive wounds associated with trauma and burns. Chronic wounds heal differently from acute wounds and as such require different treatment modalities. They have a prolonged inflammatory phase, are susceptible to infections, and are unable to respond to repair stimuli from within the wound bed. Infections are a serious threat to wound repair and if neglected can lead to systemic and life-threatening complications such as sepsis and multiple organ dysfunction, and as such control of biofilm is a key part of chronic wound management.[9–12] Infections aside, chronic wounds have a plethora of factors that contribute to delayed wound repair.[13] Critical to this process are the ability of cells to sense and respond to the changes in the microenvironment (biophysical and biochemical) of the wound. While there is an increasing emphasis on understanding the biophysical relationship to disease and wound healing, the fact that the cellular response (migration, differentiation, and proliferation) is at least in part mediated by chemokinesis is well established. However, compounding this is the fact that cells residing in chronic wounds have a lower density of growth factor receptors and lower mitogenic potential, resulting in the impaired response to environmental cues.[14–18]

Growth factors are intrinsic elements that are critical to the orchestration of events throughout the wound healing process. Within wounds, cytoactive factors are present both in the soluble form and as bound to the ECM.[19] These factors when confined to the ECM, albeit at low surface concentrations, provide localized interactions to numerous surface receptors of cells to elicit a cellular response. To account for mass transport across the space of the fluid bathing the cells of the wound surface, soluble factors may be required at greater concentrations than intrinsically immobilized factors to elicit a response and have shorter resident times. Numerous soluble cytoactive factors have been investigated for their ability to improve wound healing outcomes with platelet-derived growth factor (PDGF), fibroblast growth factor, vascular endothelial growth factor, insulin-like growth factor, transforming growth factor β (TGFβ), and epidermal growth factor (EGF)[20,21] being among the most intensively studied. While numerous growth factors promote proliferation and migration, repeated topical administration of cytoactive factors in large doses may lead to toxic adverse events including tumorigenicity.[22] In fact, over the past 2 decades, only one cytoactive growth factor, recombinant human PDGF (rhPDGF-BB; Regranex, Health-point Ltd, Ft. Worth, Tx), has been approved by the FDA for the treatment of chronic wounds,[23] which, notably, is accompanied with a “black box” warning for potentially increasing the neoplastic transformation systemically. Thus, there is an urgent need for viable treatments to promote the healing of chronic wounds.

In addition to cytoactive factors, the extracellular environment in wounded tissues is abundant in structural proteins such as procollagen, tropoelastin, fibronectin, and laminin that are abundant in native reactive groups (e.g., lysines and cysteines, which contain primary amines and sulfhdryl moieties, respectively).[24] In a previous study, we demonstrated, using ex vivo tissue explants, that polymeric beads may be tethered to these reactive groups.[25] While polymeric beads are a viable option, it requires the development of nano- or microcarriers with defined release kinetics whose byproducts, upon degradation, may adversely affect wound repair. Despite the increasing number of studies reporting about the efficacy of controlled-release technology using polymeric materials, very few have met with FDA approval or are currently being used clinically. Discussing the merits and demerits

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of controlled release is out of the scope of this study. However, we recognize that alternative methods to facilitate drug retention on wounds are required. Thus, in this study, we present a method where we conjugate growth factors directly on to the wound bed in situ and explore its potential to facilitate wound closure. We have used a variety of in vitro, ex vivo, and in vivo methods to validate and document the efficacy of such an approach.

MATERIALS AND METHODS

In Vitro Studies.

Preparation of EGF.—(i) Soluble EGF (sEGF): Human recombinant EGF (ProspecBio) was administered at 300 ng per treatment dissolved in 5% (w/v) polyethylene glycol (PEG) in a total volume of 30 μL. (ii) Crosslinked EGF: EGF was conjugated with sulfosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (s-SMCC; ThermoPierce) following manufacturer's protocol. This conjugated EGF is referred to as CxEGF in this study. The conjugation efficiency was determined by the well-established TNBS assay (ThermoPierce) to determine the reduction in free-amine groups of s-SMCC following manufacturer's protocol. (iii) Quenched EGF (qEGF): s-SMCC-conjugated EGF was incubated with 10 molar excess of β-mercaptoethanol (FisherSci) and dialyzed against PBS to remove unreactive β-mercaptoethanol. This is referred to as qEGF in this study. (iv) Vehicle control: 5% (w/v) PEG in Hank's balanced salt solution was used as vehicle control. For all experimental groups, 5% PEG was used as our vehicle.

In Vitro Surface Modification.—An in vitro model of the wound surface was generated by silanizing glass coverslips with either trimethoxy(propyl)silane (TPS) or 3-mercaptopropyl trimethoxysilane (3-MPTS). Disulfide bonds (if any) on the surface of the silanated coverslips were reduced using 10 μM tris(2-carboxyethyl)-phosphine (TCEP) for 10 min to generate an abundance of −SH bonds. These exposed −SH groups were used to covalently immobilize EGF.

Confirming the Surface Covalent Immobilization.—Prior to employing the bioconjugation approach in vivo, where the maleimide group of EGF modified with s-SMCC would be tethered to native sulfhydryl (−SH) groups on the wound bed, we first validate the chemistry in vitro. Thus, we first intend to confirm that −SH reactive groups were able to bind to maleimide groups on the crosslinker; to this effect, we used methoxyPEG (5000 mw) or methoxy PEG-maleimide (5000 mw) for these experiments. (i) Goniometry: Unmodified glass coverslips and TPS- or 3-MPTS-modified glass coverslips were treated with TCEP and incubated with methoxyPEG or methoxyPEG-maleimide for 10 min, washed thoroughly with HBSS, and air dried. Using a Ramé-Hart goniometer, the contact angles on all surfaces were determined using deionized water. (ii) Ellipsometry: Unmodified silicon wafer (Si/SiO_2) and TPS- or 3-MPTS-modified silicon wafer were treated with TCEP and incubated with methoxyPEG or methoxyPEG-maleimide for 10 min, washed thoroughly with HBSS, and air dried. Using an Elli2000 (NanoFilm technologie, Germany), ellipsometric angles (Δ and Ψ) were resolved. Measurements were taken at an incidence angle of 60°.

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Measuring Bioactivity in Vitro.—Human immortalized keratinocytes (HaCaT cells) were plated on unmodified glass surfaces or on surfaces covalently immobilized with EGF or on surfaced with qEGF, in their exponential growth phase, in Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum, and the proliferation was determined after 5 days using the MTT assay.[26] Cells were also cultured in a growth medium containing 10% serum for comparison. A flowchart illustrating the sequence is provided in Figure 2A.

Ex Vivo Feasibility Study.—Prior to the conduction of in vivo experiments and after validating our reaction chemistry in vivo, we evaluated the feasibility of the bioconjugation technique ex vivo on porcine dermal tissues, as described previously.[25] Briefly, porcine dermis tissue surfaces were treated with 10 μM TCEP for 10 min, following which amine-coated fluorescent poly(lactic-*co*-glycolic acid) (PLGA) nanospheres either natively or activated with s-SMCC were added to the surface. 30 min after incubation, the surfaces were rinsed in saline thoroughly and imaged under a fluorescent dissection microscope. The percentage area coverage of tissue with or without the modified PLGA beads was quantified.

In Vivo Studies.—All procedures adhered to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol for this experiment was approved by the Institutional Animal Care and Use Committee of University of California, Davis. A flowchart illustrating the sequence is provided in Figure 2B.

Creating Full-Thickness Splinted Dermal Wounds.—Genetically diabetic 12 week old male mice (db/db; BKS.Cg-m +/+ Leprdb; Jackson Laboratories, Bar Harbor, ME) were used for this study. These mice were chosen for our studies as they demonstrate a number of key features of human type II diabetes, including but not limited to delayed wound healing,[27–29] that are not exhibited in other diet-induced diabetic models.[30] The average ±SEM body weight of the mice was 42 ± 0.5 g. 8 mm Full-thickness wounds were created on the dorsal surface of db/db mice, as described previously.[31,32] Details pertaining to the creation of wounds and validating the splinted model for the epithelial closure have been described previously in diabetic mice in the presence or absence of a growth factor.[31,32]

Treatment of Wounds.—For the in vivo evaluation of epithelial wound closure, the various groups/treatment regimens are indicated in Table 1. Freshly wounded surfaces in mice were treated with 10 μM TCEP for 10 min following which either covalently modified human recombinant EGF (CxEGF) or qEGF, dissolved in 5% PEG, was applied onto the wound bed for 30 min prior to the application of the bandages. Mice in the sEGF group received native sEGF (300 ng) dissolved in PEG immediately after wounding and twice daily until day 10. The NoTx group remained without any treatment through the study period. All wounds were dressed using a sterile untreated glass coverslip over the splint and a semiocclusive bandage. We previously reported that use of such a splinting and bandage thickness did not adversely affect wound closure.[31,32] The thickness of the splint was approximately 2.1 mm, thus any potential contact of the wound bed to the glass coverslip or bandage was avoided. We note that a sterile coverslip was placed over the surface of the

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sutured O-ring splint to minimize introducing mechanical abrasion of the functionalized wound bed as a confounding variable. Mice in the CxEGF-6x group received TCEP and covalently modified human recombinant EGF (30 ng) once immediately after wounding and once every other day over 11 days.

In Vivo and Histological Analyses.—All analyses were performed as described previously.[31,32] Briefly, wounds were grossly evaluated and imaged daily, and the wound area was imaged and measured using an image analysis software, and percentage wound closure was calculated. Mice were humanely euthanized on day 11 and the entire wound bed was harvested. Histologic analyses were performed by a board-certified veterinary pathologist using methods described previously.[31,32] (i) Collagen: Fibrovascular dermal proliferation (granulation tissue) was measured by examining the Picrosirius red-stained sections under polarized light, which highlighted the newly deposited dermal collagen. Using the image analysis software, the wound bed area was selected, and the amount of collagen in the selected area was automatically measured and expressed as a percentage of the wound area. The type of collagen was also analyzed by dividing the wound bed into four regions: two peripheral and two central, photographed under 400× magnification. Using the image analysis software, the proportion of green (type III collagen) fibers and red-yellow-orange (type I collagen) fibers was quantified. (ii) Inflammation: The inflammatory response was assessed using a semiquantitative scoring system ranging from 0 to 4, in which 0 indicates no inflammation, 1 indicates that 0–25% of the wound area was infiltrated by inflammatory cells, 2 indicates 25–50%, 3 indicates 50–75%, and 4 indicates >75% of the wound area was infiltrated. The inflammatory response was also quantified by counting the numbers of neutrophils, lymphocytes, plasma cells, and macrophages in three 40× objective fields, two close to the wound margins and one central. (iii) Granulocytes and macrophages: GR1-positive granulocytes and F480-positive macrophages were labeled by immunohistochemistry. Using Aperio positive pixel count algorithm (Leica BioSystems AG, Germany), the total number and positively labeled cells were counted and expressed as a ratio of positive cells to total number of cells.

Detection of Crosslinked EGF on Dermal Wounds.—The persistence of CxEGF, qEGF, and sEGF in the wound bed was determined using ELISA specific to antihuman EGF. Mice were euthanized at each time point and the wound bed was harvested. The tissue was digested using 7.5 units/mL collagenase in the presence of 1 μM dithiothreitol (DTT) over 2 h at 37 °C. This was centrifuged at 14,000 rpm and the supernatant following which ELISA was performed. Specificity of the ELISA was verified using mouse recombinant EGF. The amount of EGF (mg) measured on the wound bed was expressed per surface area (mm^2) of the tissue.

Statistical Analysis.—(i) In vitro studies: For cell proliferation experiments, cell proliferation was expressed as a percentage of control cultures (cells cultured in media with 1% serum); one-way ANOVA followed by Dunnett's multiple comparison test compared with control cultures was used to determine the significance. For changes in the contact angle and thickness after PEGylation on silicon surfaces, paired t-test was used to compare the PEGylated group with the native group. (ii) In vivo studies: Prior to conduction of the

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studies, we performed power analysis for sample size estimation. We determined that a minimum of five samples would be required to obtain a power of 0.85 ($\alpha = 0.05$), comparing a minimum detectable difference in means of 20% of wound closure rates with a standard deviation of 7.5% with five study groups. Therefore, in this study, we used an "n" of 5–14 animals per group with three primary control/experimental groups. Statistical analysis was performed by use of a commercially available statistical software program (SPSS, version 18.0, SPSS Inc, Chicago, IL). Normality of the data was confirmed by the Shapiro–Wilk test. The effects of treatment, time, and interaction between time and each effect on the wound closure were evaluated by using repeated measures ANOVA for each comparison. The wound closure was also compared between groups at each time point by using Student's t-test, where repeated measures ANOVA revealed significant interaction effects. Histologic scores were compared between the groups with the Mann–Whitney U test. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Impact of the Topically Applied Growth Factor on Wound Healing.

EGF is a key growth factor involved in triggering wound re-epithelialization, one of the critical early events in wound closure, whose prolonged exposure is required for eliciting a cellular response.[33–36] We therefore chose to test whether the sEGF resulted in an accelerated re-epithelialization in diabetic mice. A full-thickness splinted wound (8 mm in diameter) was created on each heterozygous ($db^{\pm}$; BKS.Cg-$m^{+/+}$ $Lepr^{db}$) mouse, as described previously.[31] sEGF (0.001% (v/v); 300 ng per 0.5 cm^2 wound), topically administered twice daily, to full-thickness splinted wounds did not significantly alter wound healing or epithelial closure in diabetic mice (db/db; Figures 3A, S1). The functional bioactivity of EGF was determined in vitro using spontaneously transformed keratinocytes (HaCaT cell line) from histologically normal skin (Figure S2). Indeed, sEGF showed potent increases in HaCaT cell proliferation when used between 6.25 and 50 ng/mL compared with untreated control cells. With this, we inferred that the lack of any benefit in wound healing in vivo was not due to EGF's bioactivity but may have been due to factors within the wound bed that inhibited retention or contributed toward the potential degradation of the growth factor. These results were consistent with a previous study from our group, where no differences in epithelial wound closure were observed with the topical treatment of PDGF-BB.[32] As a possible contributor to the lack of significant positive effect, we observed a rapid loss of recombinant human EGF from the surface of wounds within 1 h of topical administration (Figure 3B). The efficient delivery of cytoactive-factor-based therapeutics can be affected by the wound exudate, high concentrations of matrix metalloproteinases (MMPs), and absorbent bandages wicking soluble factors away from the wound surface. We hypothesized that the failure of EGF to promote wound healing was directly related to its rapid disappearance from the wound surface and that strategies to increase the retention of EGF on the wound surface would promote re-epithelialization.

A number of strategies, including biofunctionalized scaffolds, micro-/nanoparticles, and cytokine gels, have been investigated as adjunctive therapeutics for the acceleration of wound healing and the reduction of bioburden,[37–42] though very few are commercially

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available. The covalent immobilization of cytoactive factors to polymeric/metallic substrates improves the retention and bioavailability of molecules to cells and has demonstrable success in in vitro approaches.[25,43–46] However, these involve complex scaffold fabrication methodologies and subsequent surface modification steps that are time-consuming and expensive and as such have yielded very little gain. More recently, Martino et al. reported engineered growth factors with super-affinity to the native ECM, which yet again involves complex syntheses.[47] Therefore, we developed a simple delivery method that exploits the surface chemistry of the native wound bed for anchoring growth factors. Particularly, we demonstrate that cytoactive factors, such as EGF, can be covalently integrated directly into the wound surface, without the use of any biomaterial carriers, to accelerate re-epithelialization, a new paradigm for the delivery of cytoactive and antimicrobial therapeutics to improve wound healing outcomes.

Validation and Quantification of Covalently Immobilized EGF.

To achieve the immobilization of growth factors, we used sulfosuccinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (Sulfo-SMCC) as a water-soluble heterobifunctional crosslinker with an amine-reactive NHS ester and a sulfhydryl-reactive maleimide group. The native dermis presents an environment rich in cysteines and disulfides[25] that can be exploited to immobilize growth factors chemically modified with sulfo-SMCC. The feasibility to tether sulfo-SMCC-modified PLGA nanospheres to porcine dermis tissue was first evaluated (Figure S3) ex vivo. Consistent to our previous study,[25] we observed a significant increase in the attachment of the modified beads, demonstrating the successful usage of thiol chemistry.

Next, full-thickness splinted wounds (8 mm in diameter) were created on heterozygous (db$^{+/-}$; BKS.Cg-m$^{+/+}$ *Lepr*^{db}) mouse as described above. Wound surfaces were treated with a reducing agent [tris(2-carboxyethyl)phosphine; TCEP] to break the disulfide bonds. The crosslinkers and reducing agents were used at nontoxic doses; toxicity profiles of compounds were determined in vitro (Figure S4). This facilitated the exposed sulfhydryl groups for attachment to the maleimide group from s-SMCC-modified EGF (here forth referred to as CxEGF; Figure 4A). The fresh wound surface was treated with 10 μM TCEP (40 μL), following which s-SMCC-EGF (30 ng in 30 μL of 5% PEG) was crosslinked onto the wound surfaces (denoted as CxEGF). Immediately after the treatment, wounds were covered with a semiocclusive dressing to minimize scab formation.[31] Wound beds were harvested at various time points and the presence of EGF attached on the surfaces was determined by ELISA (Figure 4B). Covalently tethering EGF to the wound surface resulted in a sustained surface immobilization of the growth factor over 2 days. A gradual decrease in the amount of surface-immobilized EGF was observed after 2 days. This apparent surface coverage (2 pg/mm^2, an equivalent of 1.99×10^8 molecules/mm^2) was observed to be 1/100th of a theoretical monolayer (2.19×10^{10} molecules/mm^2 equivalent to 213.6 pg/mm^2) of EGF.[48] We note that the number of disulfide bonds available for anchoring CxEGF is difficult to ascertain in vivo and may thus contribute to the partial surface coverage. Combined with the observation that EGF was detectable on the wound surface for a maximum duration of 2 days, we speculate that the growth factors are either degraded by this time or the matrix on the surface was microanatomically renewed or a combination of

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both events may have transpired. Answering this particular question requires further investigations that are beyond the scope of the present study.

Nevertheless, secondary validation of the linkage chemistry was done using an in vitro model of the surface chemistry using (3-mercaptopropyl)trimethoxysilane (MPTS) or TPS and maleimide by a reduction in the contact angle and increased thickness of deposition (Figure 4D). The mercapto group on the MPTS-simulated sulfhydryl groups presents on a native wound bed, and TPS was devoid of any functional group simulating a passivated wound surface. In conjunction with the significantly decreased contact angle, the marked increase in the thickness demonstrated the successful attachment of PEG-maleimide (a hydrophilic polymer) to the mercaptosilane. Next, we aimed to determine if the bioactivity of EGF was retained with the linkage chemistry. For this, the bioactivity of covalently immobilized CxEGF, on MPTS- or TPS-coated glass substrates was confirmed using an in vitro cell-based bioassay (MTT), wherein cell proliferation significantly increased greater than twofold after 3 days of treatment (Figure 4C). In fact, the proliferation of cells cultured in 1% serum on surfaces with immobilized EGF were only 20% lesser than those observed when cells were cultured without exogenous EGF in the presence of 10% serum, demonstrating the potent bioactivity of immobilized EGF. This undoubtedly confirmed that the modification of EGF with s-SMCC or tethering to a surface using the thiol group was efficient in maintaining its bioactivity critical for cell proliferation and/or migration.

Covalently Immobilized EGF Promotes Wound Healing in Vivo.

Full-thickness splinted wounds were created as previously described,[31] pre-treated with TCEP, and treated with either (i) soluble topical EGF, (ii) crosslinker control (SMCC), (iii) s-SMCC-modified EGF with its maleimide group quenched with β-mercaptoethanol (qEGF), or (iv) s-SMCC-modified EGF (CxEGF). Initial experiments were performed with immobilization performed only once (CxEGF-1x), immediately after wounding, and no significant effects on wound closure rate (reduction of wound radii) were observed (Figures 5C, S5) despite the documented retention of EGF for 2 days (Figure 4B). Therefore, to prolong the exposure to EGF, wounds were treated with CxEGF every other day (CxEGF-6x) or every 4 days (CxEGF-3x) after disulfide bonds were reduced using TCEP at each time point. This targeted delivery of the growth factor resulted in a significant acceleration of the wound closure between 5 and 11 days after wounding when CxEGF was applied every other day in comparison with all other groups (Figure 5A,B). The covalent application of EGF every 4 days resulted in a similar decrease in wound radii as every other day treatments (Figure 5C). Next, we intended to confirm that the increase in the rate of re-epithelialization was due to the covalent immobilization of s-SMCC-EGF via its maleimide group. For this, mice (in a separate group) were treated with s-SMCC-EGF, whose maleimide groups were quenched with β-mercaptoethanol (qEGF). Indeed, the qEGF treatment was ineffective in altering wound healing rates and was comparable to those of sEGF or other negative control groups, confirming that CxEGF was bioactive when covalently immobilized on wounds. Histological analyses demonstrated dramatic decreases in the epithelial gap (Figure 6A) after treatment with CxEGF-6X, with comparable collagen content (Figure 6B) and reduction in inflammation (Figure 6C) in comparison with qEGF or no treatment or sEGF. Further studies failed to show a statistically significant difference in

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the numbers of F480-positive macrophages or GR1-positive granulocytes (Figure S6). No significant differences in cellularity or dermal material were observed either demonstrating a lack of toxicity in vivo. Of great relevance in the clinical use of highly expensive therapeutics such as growth factors is the greatly reduced amount of test article required to achieve therapeutic benefit. The total amount of EGF administered to the wound surface by the covalent linkage of CxEGF-6X is calculated to be 180 ng, CxEGF-3X is calculated to be 90 ng, and for topical administration of sEGF 7200 ng was delivered over the 11 days. Yet, the rate of increased re-epithelialization was over 20 % greater for the CxEGF-6X group than the sEGF group (Figure 7). We note that preclinical studies that preceded the approval of the only growth factor for wound healing (PDGFbb-Regranex/Becaplermin) showed only about a 20% improvement in wound healing over control.[23,49–53] Furthermore, a phase IV clinical study on the efficacy of Regranex demonstrated that the topical administration of rhPDGFbb promoted wound closure in 43% of diabetic ulcers, although the rate of healing was no better than 13%.[6,54] Since there are no therapies, to the best of our knowledge, that improve healing significantly higher at this time, our strategy to promote re-epithelialization using covalent linkage chemistry is a significant improvement that is clinically important.

Limitations.

The report herein establishes proof of concept that the covalent linkage of growth factors such as EGF promotes wound closure. While results are encouraging, the study is not without limitations. EGF was chosen due to its unambiguous ability to promote epithelial closure in vitro and data documenting its ability to stimulate epithelial migration when immobilized using linkage strategies.[43] While results reported herein are encouraging, many soluble growth factors with encouraging results in vitro and in animal studies have failed to translate to the clinic. The failure/success of the translation may in part be related to the method of delivery, and here, we report a unique method that has potential for clinical success. Using covalent immobilization, we demonstrate the relatively prolonged retention of EGF on the wound surface. However, due to the large abundance of cysteine and primary amine residues on a wound surface[24,25] from cells and matrix proteins, it is nearly impossible to determine with great accuracy the molecule(s) on the wound bed to which CxEGF was covalently bound to. Future studies will be required to develop the techniques to investigate the mechanisms of such attachment and the molecular mechanisms by which EGF that was retained facilitated wound healing.

While PDGF has been shown to have tumorigenic potential, EGF/EGF receptor (EGFR) signaling is also implicated in various types of cancer and carcinogenesis[55,56] by promoting cell proliferation and differentiation. While we did not observe any tumorigenic/toxic potential of immobilized EGF, albeit when used at significantly low concentrations, in our mouse wounds, its effects with chronic administration needs to be further evaluated. This is especially important considering we utilized TCEP, a known toxin/carcinogen at high concentrations,[57,58] to chemically reduce the surface of the wounds and exposure −SH residues. Careful follow-up studies are required to establish the safety profile of the approach we report. Next, we evaluated the efficacy of the covalent immobilization approach in sterile wounds. It is well recognized that wound healing and repair are adversely affected by the formation of bacterial biofilms.[10,59] In this study, no bacteria were added to create an

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infected wound in order to limit the number of confounding variables while evaluating efficacy. However, we recognize that future studies focusing on evaluating our approach in nonsterile wounds may be important. Next, we recognize that we utilized splinted wounds in mice, a species that heals primarily by wound contraction in the absence of splinting, to evaluate epithelial wound closure efficiency. Since we only perform our experiments until we observed total re-epithelialization, subsequent changes in the granulation tissue and stromal reorganization beyond the 11 days were not investigated. A focus on the efficacy and safety in large animal models are critical before entering human clinical trials. Furthermore, we recognize that we use a glass coverslip to cover the splinted wound. Considering impairment in cellular metabolism in diabetes, it is possible that the respiration and gas exchange of cells at the wound surface could be altered using our approach, although our prior studies comparing cover-slipped wounds to noncovered ones did not reveal cover-slip-related healing impairments.[31] In this study, we did not evaluate the effect of covering wounds with coverslips on oxygen fluxes or metabolism. While enabling us to minimize the introduction of confounding variables associated with the mechanical removal of immobilized EGF (from bandage contact with the wound surface), it is possible that our approach introduced other confounding variables through altering the cellular metabolism. Recent studies from our group and others demonstrate the importance of oxygen flux and the activation of the hypoxia-inducible factor 1α in wound repair/regeneration.[60–65] Chaperone and stress proteins may be activated by oxidative stress, a major contributor to delayed healing in diabetic wounds.[66] Whether the use of an antioxidant (reducing agent such as TCEP) modifies surface reactive oxygen species to facilitate EGF attachment can only be speculated and requires further investigation. Likewise, the effects of such agents on modulating enzymatic systems (e.g., MMPs and TIMPs) are out of the scope of this study. Due to the inherent complexities of multiple signaling pathways implicated and the intrinsic challenges of measuring oxygen fluxes in the wound bed, these were not attempted in this study.

CONCLUSIONS

The above results point to a new and easily implemented approach for the delivery of therapeutic agents. Essentially, the approach changes the microenvironment of the wound to promote wound closure rather than treating the wound with soluble agents or introducing macroconstructions of biomaterials. The results document the efficacy of using direct covalent immobilization to improve wound healing outcomes. We have demonstrated the efficacy of integrating EGF into the wound bed, but the positive in vivo results suggest a broad application of this approach. The approach is generalizable and holds promise for integrating cytoactive factors, antimicrobials, analgesics, anesthetics, and ECMs to the patient's benefit. We note that this approach has numerous advantages including (1) markedly decreased treatment frequency. This will reduce discomfort associated with frequent bandage changes and increase "patient" compliance. It is even foreseeable that protocols will be simplified to enable in-home treatment. (2) Decreased cost of (sometimes very costly) agents used due to the markedly decreased total amount of agent expended. (3) Ability to utilize a wide array of commercially available agents. (4) Decreased adverse

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events predicted as the total amount of local and systemic exposure to potent cytoactive agents is much less than with frequent topical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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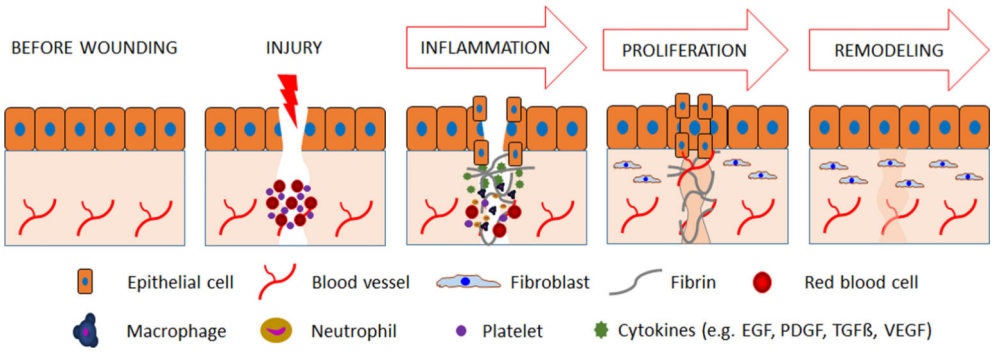

Figure 1.

Schematic of events during wound repair. Hemostasis begins on the onset of injury and is followed by the inflammatory phase. During inflammation, debris and pathogens are removed, accompanied by the secretion of growth factors to facilitate wound repair. This is followed by the proliferative phase, which includes the formation of a fibrin clot, re-epithelialization, and contraction of the wound margin. The last phase involves remodeling, where the matrix reorganizes and the wound is fully closed.

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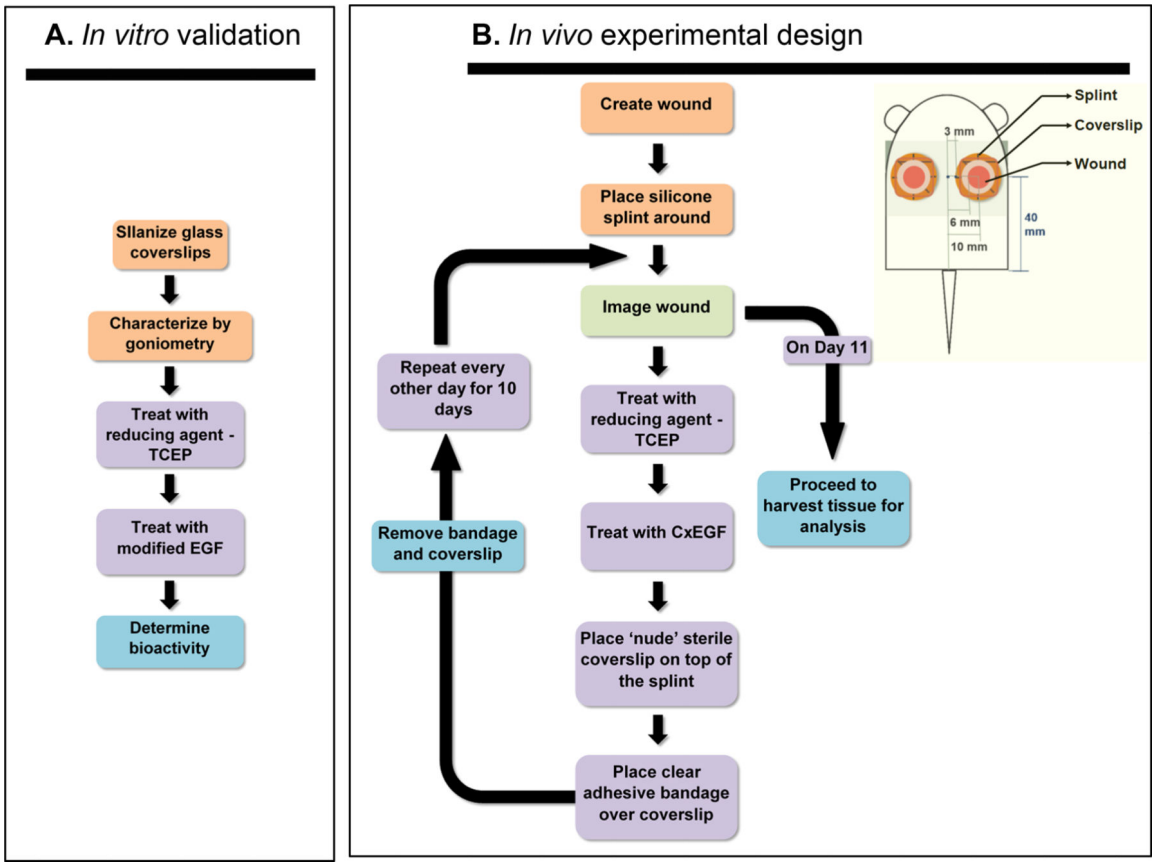

Figure 2.

A flow chart, for the experimental design, (A) validating the linkage chemistry in vitro and (B) conduction of in vivo wound closure experiments in a full-thickness splinted wound model using diabetic mice is illustrated.

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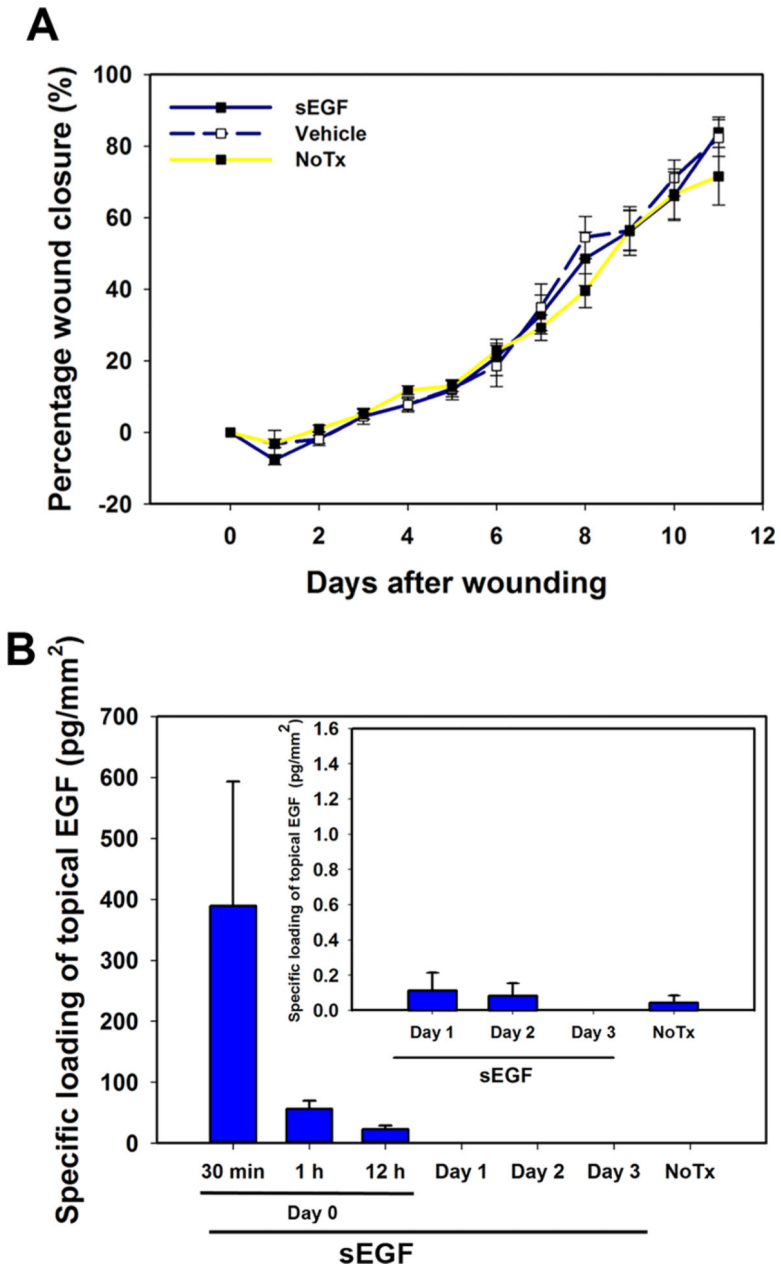

Figure 3.

(A) Topically applied sEGF did not promote epithelial closure. Percentage wound closure in mice with splinted full-thickness wounds (8 mm diameter) treated without any treatment (NoTx) or with either sEGF or vehicle over 11 days. No significant effect of group or interaction between group and time was observed ($P > 0.24$, repeated measures ANOVA). No significant differences between groups were observed for any time point ($p > 0.05$, Kruskal–Wallis test). (B) sEGF is rapidly removed from the wound surface. Persistence of sEGF in the wound bed as determined by ELISA specific to antihuman EGF. Amount of EGF measured on the wound bed is expressed as mass per mm^2 area of the tissue.

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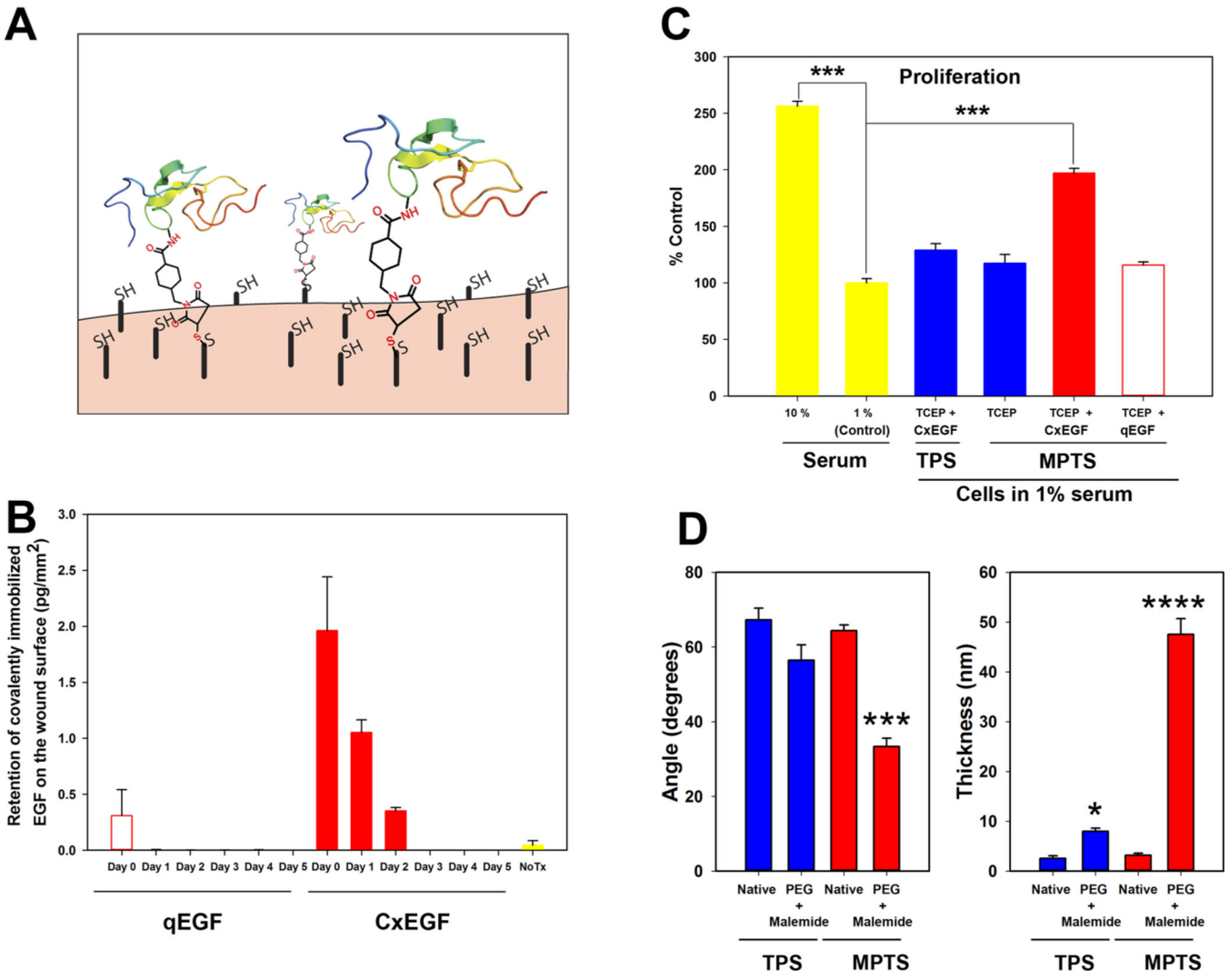

Figure 4.

Crosslinking EGF to biological surfaces using thiol chemistry. (A) Representative schematic of the chemistry employed for the covalent immobilization of EGF onto the wound bed. Covalently modified growth factors (e.g., EGF) using heterobifunctional crosslinkers are linked to disulfide groups on the wound surface. Free sulfhydryl groups on the wound surface are exposed using a potent yet nontoxic dose of a reducing agent. (B) Cross linking increases the persistence of EGF on the wound surface in vivo. Persistence of crosslinked EGF (CxEGF) and reactive-moiety qEGF in the wound bed as determined by ELISA specific to antihuman EGF. The amount of EGF measured on the wound bed is expressed as mass per area of the tissue. (C) Covalent immobilization of EGF strongly promotes proliferation in vitro. HaCaT cells were cultured in a growth medium containing 1% (v/v) serum (FBS). The proliferation rates are expressed as % control when cultured on surfaces [TPS or 3-MPTS] immobilized with EGF. As positive control, cells were also cultured in the growth medium containing 10% (v/v) FBS. ***$p < 0.001$, one-way ANOVA followed by Dunnett's multiple comparison test compared with control cultures (cells cultured in media with 1% serum). (D) Changes in the contact angle and thickness of silanized surfaces after

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immobilization with maleimide-conjugated PEG demonstrating the proof of covalent linkage chemistry. *$p < 0.05$ and ****$p < 0.0001$, and paired *t*-test was used to compare differences between the control and treated groups for each chemistry.

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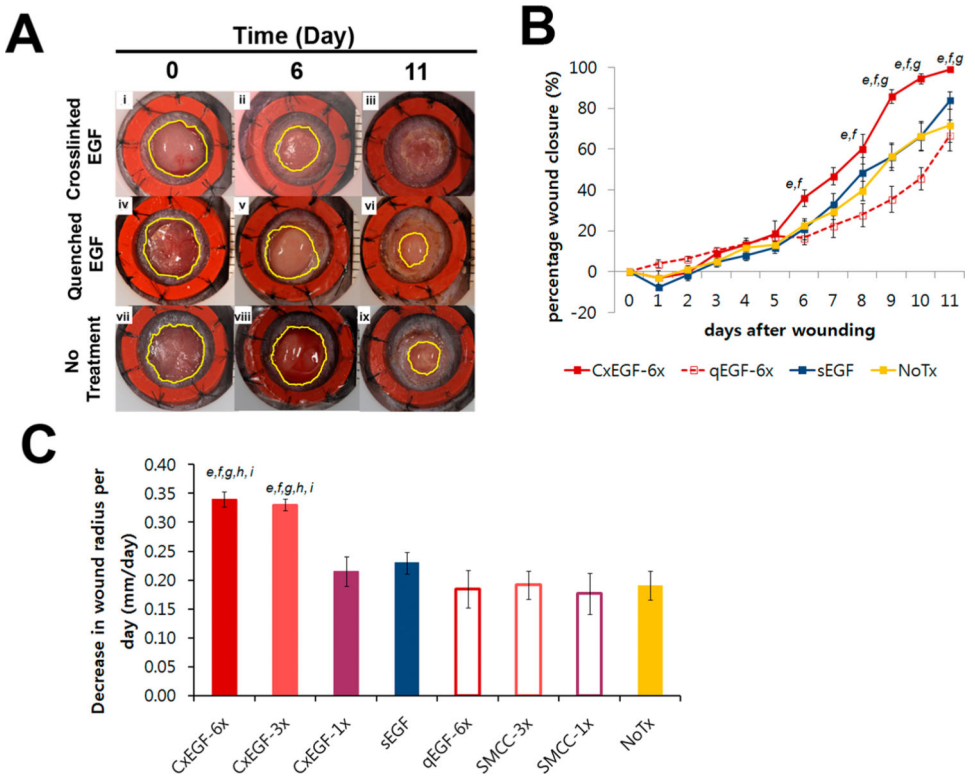

Figure 5.

Covalent immobilization of EGF in diabetic wound beds promotes epithelial wound closure. (A) Images of representative animals from the groups of CxEGF-6x, sEGF, and NoTx, which were taken on days 0, 6, 11. (i) CxEGF-6x, day 0; (ii) CxEGF-6x, day 6; (iii) CxEGF-6x, day 11; (iv) sEGF, day 0; (v) sEGF, day 6; (vi) sEGF, day 11; (vii) NoTx, day 0; (viii) NoTx, day 6; and (ix) NoTx, day11. (B) Crosslinked EGF treated six times over 11 days (CxEGF-6x) group had significantly improved wound healing compared to the sEGF delivered topically twice daily, no treatment (NoTx), and qEGF-6x groups. [[e]The CxEGF-6x group had a significantly higher percentage wound closure compared to the NoTx group ($P < 0.01$). [f]The CxEGF-6x group had a significantly higher percentage wound closure compared to the qEGF group ($P < 0.01$). [g]The NoTx group had a significantly higher percentage wound closure compared to the qEGF group ($P < 0.04$)]. (C) The groups of CxEGF-6x and CxEGF-3x had significantly higher wound closure rates (decrease in wound radius per day) compared to the rest of the groups including the sEGF group ($P < 0.05$).

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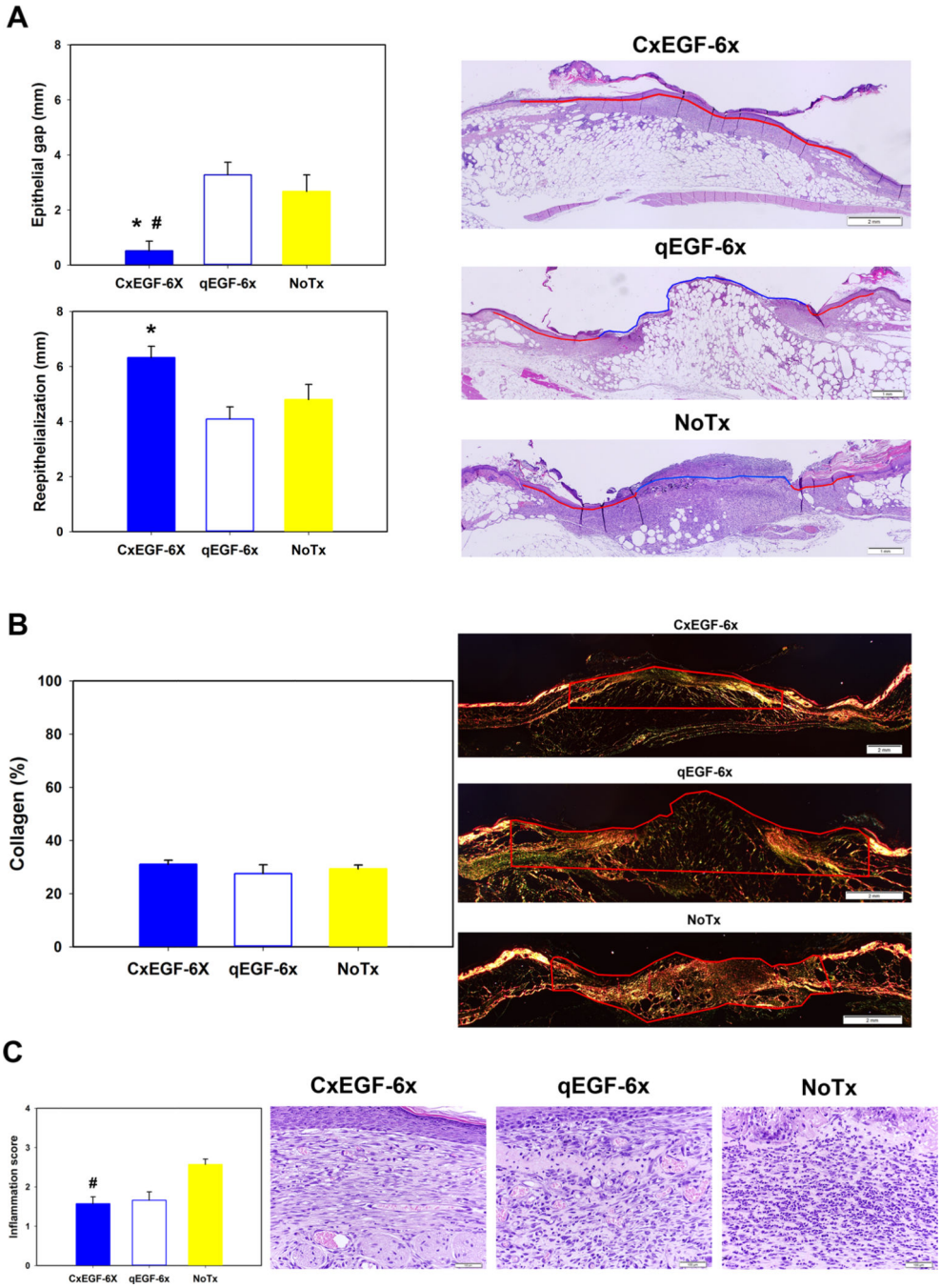

Figure 6.

Histopathological evaluations of a wound bed from a db/db mouse treated with/without crosslinked EGF over 11 days upon wounding. Scale bars = 500 μm. (A) Measurement of the epithelial gap (blue line) and re-epithelialization (red line). The epithelial gap was defined as the distance between the advancing edges of the keratinocyte migration measured in millimeters. The length of re-epithelialization was defined as the length of the layer of proliferating keratinocytes covering the wound area. This value was obtained by measuring the distance between the free edge of the keratinocyte layer and the base, where the cells

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were still associated with native, non-affected dermal tissue, and the final value was the sum of the distance in millimeters of both sides. H&E staining. Graphs indicate the mean ± standard deviation of the epithelial gap/re-epithelialized measure on surface wounds after 11 days with or without treatments. (B) Measurement of the fibrovascular dermal proliferation in the wound bed. Using picrosirius red stain under polarized light, the bright collagen fibers of the wound bed are highlighted and automatically measured by the software. The final data are expressed as a percentage of the outlined wound area comprising collagen. Graphs indicate the mean ± standard deviation of % collagen measured in surface wounds after 11 days with or without treatments. (C) Crosslinked EGF elicited less inflammation. The inflammatory response was assessed using a semiquantitative scoring system ranging from 0 to 4, where 0 indicates no inflammation, 1 indicates 0–25% of the wound area affected, 2 indicates 25–50% of the wound area affected, 3 indicates 50–75% of the wound area affected, and 4 indicates >75% of the wound area affected. H&E staining. Graphs indicate the mean ± standard deviation of the inflammation score. *$p < 0.05$ compared with qEGF-6x and #$p < 0.05$ compared with NoTx (t-test/Mann–Whitney rank sum test).

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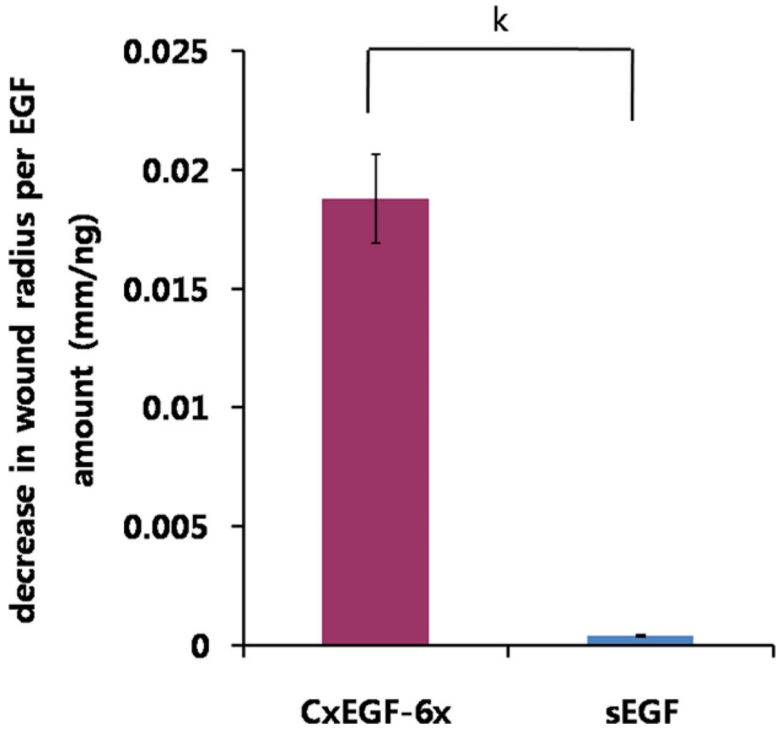

Figure 7.

Wound closure rate per applied EGF amount in the CxEGF-6x group was about 50 times higher than that in the sEGF group, which was statistically significant ($^{k}p < 0.001$). This indicates that a significantly less amount of test article can be utilized with the improved therapeutic effect.

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Table 1.

Treatment Regimen Employment for in Vivo Re-Epithelialization

						EGF		
group	**no. of Tx**	**frequency**	**TCEP**	**SMCC**	**PEG**	**crosslinked**	**soluble**	**no. of animals**
CxEGF-6x	6	every 2 days	×	×	×	×		7
qEGF-6x	6	every 2 days	×	×	×	quenched		9
CxEGF-3x	3	every 4 days	×	×	×	×		8
SMCC-3x	3	every 4 days	×	×	×			11
CxEGF-1x	1	once at the time of wounding	×	×	×	×		8
SMCC-1x	1	once at the time of wounding	×	×	×			5
sEGF	22	twice a day			×		×	11
vehicle	22	twice a day			×			9
NoTx	0							14

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