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# Bioresponsive Dextrin−rhEGF Conjugates: *In Vitro* Evaluation in Models Relevant to Its Proposed Use as a Treatment for Chronic Wounds

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**Abstract:** We recently developed a bioresponsive dextrin-recombinant human epidermal growth factor (rhEGF) conjugate as a polymer therapeutic with potential for use in the promotion of tissue repair. The aim of these studies was to use patient-derived wound fluid and fibroblasts to evaluate its potential for further development as a treatment for chronic wounds, such as venous leg ulceration, a growing clinical challenge in the aging population. First, the levels of EGF (ELISA assay), α-amylase and elastase (enzyme assays) were measured in patient-derived acute and chronic wound fluid. EGF was detected in acute, but not in chronic wound fluid. α-Amylase concentrations were higher in acute (188 IU/L), compared to chronic wound fluid (52 IU/L), but both were in the range of human serum levels. Although elastase was present in chronic wound fluid (2.1 ± 1.2 RFU/min), none was detected in acute wound fluid. Dextrin−rhEGF incubation in chronic wound fluid led to endogenous α-amylase-mediated release of rhEGF (ELISA) that was maximal at 48 h. When the migration of HaCaT keratinocytes and of human fibroblasts (isolated from patient-matched, normal skin and chronic dermal wounds) was studied *in vitro* using the scratch wound assay, enhanced cell migration was observed in response to both free rhEGF and α-amylase-activated dextrin−rhEGF conjugate compared to controls. In addition, fibroblasts displayed increased proliferation (normal dermal fibroblasts ∼160%; chronic wound fibroblasts ∼140%) following incubation (72 h) with dextrin−rhEGF that had been exposed to physiological levels of α-amylase (93 IU/L). These results suggest further preclinical *in vivo* evaluation of dextrin−rhEGF is warranted to determine whether conjugate pharmacokinetics and rhEGF liberation into such a complex and aggressive environment can still lead to bioactivity.

**Keywords:** Polymer therapeutics; chronic wounds; dextrin−epidermal growth factor conjugate; fibroblasts; keratinocytes

## Introduction

Chronic ulceration of the lower limb presents a major clinical challenge. In Western countries incidence is rising due to the aging population and an increase in risk factors such as smoking, obesity and diabetes.[1] Prevalence in the U.K. population aged >60 years is 3%,[2] and in the US 15%, a figure predicted to rise to 25% by 2050.[3] The lack of

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adequate treatments results in an extremely poor quality of life.[4] Impaired dermal wound healing is characterized by the persistence of chronic inflammatory cells, the disordered synthesis and remodeling of the extracellular matrix (ECM), and a lack of re-epithelialization.[5] A number of growth factors including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factor-$\beta$ (TGF-$\beta$) act in concert to promote wound repair,[6−8] but their levels can be significantly reduced in chronic wound exudates[9,10] due to proteolytic degradation and/or denaturation by reactive oxygen species (ROS).[9,11−13]

Although polymer therapeutics have found increasing clinical use,[14−16] they have only recently emerged as new tools to promote tissue repair[17] and for tissue protection.[18] We have developed a novel concept called polymer-masking-unmasking-protein therapy (PUMPT)[19] that uses a biodegradable polymer (e.g., dextrin[19,20] and hyaluronan[21]) to transiently mask protein activity, thus stabilizing/inactivating the protein during transit. In the presence of an enzyme able to trigger polymer degradation, protein "unmasking" occurs and bioactivity is restored. Recently we applied this concept to the design of a dextrin-recombinant human epidermal growth factor (rhEGF) conjugate, to determine whether such an approach might have potential for application in tissue repair.[22] Dextrin, α-1,4 poly(glucose), is a biocompatible polymer used clinically as a peritoneal dialysis solution,[23] and it is particularly useful for PUMPT as it is readily degraded by α-amylase, an enzyme which is widely distributed in extracellular fluids. A first proof of concept study[22] showed that dextrin conjugation could protect rhEGF against degradation by proteinases (including the clinically important wound protease neutrophil elastase), and proliferation assays, involving the high epidermal growth factor receptor (EGFR)-expressing epidermoid carcinoma cell line (HEp2) and HaCaT keratinocytes as models, showed that whereas polymer conjugation reduced activity, exposure to physiological concentrations of α-amylase led to time-dependent restoration of bioactivity that was prolonged over 8 days with HEp2 cells. Moreover, the enzyme-activated conjugate


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induced phosphorylation of EGFR in HEp2 cells suggesting that, like rhEGF, it acts by stimulation of signal transduction pathways.[22]

As the ultimate goal of this project is to develop a conjugate suitable for clinical evaluation, the aim of these studies was to further investigate the biological properties of dextrin−rhEGF in models more relevant to the clinically setting. First, it was necessary to establish the levels of EGF, elastase and α-amylase in human wound fluid samples taken from chronic and acute wounds. The ability of dextrin−rhEGF to liberate rhEGF in chronic wound fluid was determined. As a key step in wound repair is induction of local cellular responses in keratinocyte and fibroblast cellular populations, these were modeled *in vitro*. Potential for re-epithelialization was studied by examining the effect of dextrin−rhEGF (±α-amylase) on the *in vitro* proliferation (MTT assay) and migration (scratch wound assay) of human fibroblasts that were isolated from patient-matched normal skin or a chronic dermal wound. The effect of the dextrin−rhEGF conjugate on HaCaT keratinocyte migration was also studied *in vitro* using the scratch wound assay.

## Materials and Methods

**Materials.** rhEGF was from Prospec-Tany Technogene Ltd., (Rehovot, Israel) and dextrin ($M_w \sim 42{,}000$ g/mol) from ML Laboratories (Liverpool, U.K.). The human, EGF Quantikine ELISA kit was from R&D Systems (Minneapolis, MN). 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT), human neutrophil elastase, bovine serum albumin (BSA) and human salivary α-amylase, were from Sigma-Aldrich (Poole, U.K.). The Phadebas amylase assay kit was from Magle Life Sciences (Lund, Sweden) and the EnzCheck elastase assay kit was from Molecular Probes (Invitrogen, Paisley, U.K.). The DC protein assay and electrophoresis equipment was purchased from Bio-Rad Laboratories (Hemel Hemstead, U.K.). All other chemicals were of analytical reagent grade.

Human HaCaT keratinocytes (a spontaneously immortalized human keratinocyte cell line),[24] and fibroblasts derived from biopsies of the edge of a chronic venous leg ulcer and taken from the ipsilateral thigh of the same patient that were hTERT (human telomerase, reverse transcriptase) immortalized, were kindly donated by Dr. Ivan Wall and Dr. Matthew Caley (Wound Biology Group, Tissue Engineering and Reparative Dentistry, Cardiff University). Epilife medium and HKGS kit (bovine pituitary extract, bovine insulin, hydrocortisone, bovine transferrin) were from Cascade Biologics (Mansfield, U.K.). Dulbecco's minimum essential media (DMEM), Ham's F12 nutrient media, trypsin−EDTA, penicillin G, streptomycin sulfate, amphotericin B, hydrocortisone, adenine, cholera toxin, insulin and fetal calf serum (FCS), were all from Invitrogen (Paisley, U.K.).

**Dextrin−rhEGF Conjugates.** The dextrin−rhEGF conjugates used in these experiments were synthesized and characterized as previously described.[22,25,26] The conjugates used had a mean protein content of 16.3 ± 4.4 wt % and a free rhEGF content of <1%.[22]

**Wound Fluid Collection and Analysis.** After Local Research Ethical Committee approval, informed patient consent was obtained for all procedures. Patients undergoing mastectomy surgery (acute wound fluid) or with venous leg ulcers (chronic wound fluid) were recruited from inpatient and outpatient populations at the University Hospital of Wales, Cardiff, U.K.

Acute wound fluid samples were obtained over a 24 h period using perioperatively placed suction drains (Jackson-Pratt drains; Cardinal Health, Dublin, Ohio). Samples (1 mL) were clarified by centrifugation (15000*g*, 5 min at 4 °C) and supernatants stored at −80 °C until use.

To collect chronic wound fluid each venous leg ulcer was dressed with Release (Johnson & Johnson Wound Management Ltd., Gargrave, U.K.) and covered with a Bioclusive (Johnson & Johnson) film dressing. These dressings remained *in situ* >4 h. When the wound dressing was saturated, the film was removed and the saturated dressing removed using sterile forceps and placed in a sterile container on dry ice for transfer to a −80 °C freezer. Nonsaturated regions of the dressings were removed with sterile scissors, and wound fluid eluted by immersion of the dressing in wound fluid elution buffer (0.1 M Tris-HCl buffer, pH 7.4, containing 0.1% (v/v) Triton X-100), using 500 μL of buffer/cm$^2$ dressing. The dressings were left for 2 h at 4 °C under constant agitation.[27] The eluted wound fluid samples were then aliquoted (200 μL) and stored at−80 °C until use.

*The protein content* of the wound fluid samples was determined using the Bio-Rad DC protein assay with BSA as a standard. Standards and samples (20 μL; in triplicate) were added to a 96-well microtiter plate and assayed as per the manufacturer's instructions. After 15 min, absorbance at 620 nm was measured using a Fluostar Optima microtiter plate reader (BMG Labtech, Aylesbury, U.K.). Protein content was determined from the calibration curve.

α-*Amylase activity* was measured, as previously described.[28] Each wound fluid sample and a blank of elution buffer (200 μL) was added to 4 mL of double-distilled water

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***Table 1.*** Analysis of Patient Acute and Chronic Wound Fluid Samples[a]

| | | | α-amylase activity | | elastase activity | |
|---|---|---|---|---|---|---|
| wound fluid | patient | protein content (mg/mL) | (IU/L)* (mean ± SD) | (IU/L/mg protein) (mean ± SD) | (RFU/min)** (mean ± SD) | (RFU/min/mg protein) |
| acute | 1 | 4.28 | 172 ± 19 | 201 ± 22 | nd | nd |
| | 2 | 4.13 | 166 ± 34 | 201 ± 41 | nd | nd |
| | 3 | 4.47 | 256 ± 27 | 286 ± 30 | nd | nd |
| | 4 | 4.57 | 261 ± 35 | 286 ± 38 | nd | nd |
| | 5 | 6.33 | 83 ± 39 | 66 ± 30 | nd | nd |
| chronic | A | 1.42 | 48 ± 2 | 168 ± 7 | 1.93 ± 0.18 | 13.7 |
| | B | 1.15 | 51 ± 4 | 221 ± 17 | 1.37 ± 0.37 | 11.8 |
| | C | 1.40 | 55 ± 5 | 195 ± 18 | 4.28 ± 0.54 | 30.7 |
| | D | 1.67 | 57 ± 3 | 169 ± 90 | 1.42 ± 1.24 | 8.5 |
| | E | 0.74 | 49 ± 8 | 332 ± 55 | 1.52 ± 0.31 | 20.3 |

[a] *Measured using 200 μL samples. **Measured using 100 μL samples.

($ddH_2O$), in triplicate, and prewarmed at 37 °C for 5 min. A Phadebas tablet was added to each sample, vortexed for 10 s and reincubated at 37 °C for 15 min. Then, 1 mL of 0.5 M sodium hydroxide was added to terminate the reaction and the samples were vortexed and centrifuged (1500*g*, 5 min). The absorbance of each supernatant was assayed spectrophotometrically at 620 nm (Cary 1G UV−visible spectrophotometer, Varian Inc., Palo Alto, CA), and activity determined from the Phadebas batch-matched, standard curve.

*Elastase activity* was measured using the EnzChek elastase assay in accordance with the manufacturer's instructions. The reaction buffer and substrate solution were mixed in equal quantities and added to a black 96-well analytical plate (Greiner Bio-One). Wound fluid samples (100 μL) or porcine pancreatic elastase standards (100 μL, Molecular Probes diluted from a 50 U/mL stock in $ddH_2O$ to give elastase concentrations ranging between 0.004−0.25 U/mL) were added to the reaction mixture. Fluorescence was measured with a Fluostar Optima microtiter plate reader (excitation 485 nm, emission 520 nm) at timed intervals over 1 h. Activity was determined from the calibration curve.

*The EGF content* of wound fluid samples was determined using the human EGF Quantikine ELISA assay as detailed below.

**rhEGF Release from the Dextrin−rhEGF Conjugate in Chronic Wound Fluid.** rhEGF (250 pg/mL) and dextrin−rhEGF (250 pg/mL rhEGF-equiv) were diluted in the chronic wound fluid sample having the highest elastase activity (6 mL; Patient C; Table 1) and incubated at 37 °C for 72 h. Aliquots (600 μL) were taken over time and snap-frozen, prior to storage at −80 °C. The EGF content of each sample was determined using the human EGF Quantikine ELISA assay. A 96-well plate was supplied precoated with a monoclonal antibody to human EGF. Experimental samples and aliquots of each acute and chronic wound fluid samples (200 μL; in triplicate) were added to the plate and assayed against standards of rhEGF of known concentration, as per manufacturer's instructions. The absorbance of each well was measured using a Fluostar Optima microtiter plate reader at 450 nm with wavelength correction at 540 nm, and rhEGF content was determined using the calibration curve.

**Cell Proliferation Assay.** HaCaT keratinocytes, and normal dermal and chronic wound fibroblasts were routinely cultured as described in the Supporting Information. Before undertaking the proliferation assays it was necessary to optimize the culture conditions to determine the lowest possible concentration of fetal calf serum to ensure minimal effects of endogenous EGF (see the Supporting Information). It was concluded that the proliferation assays could be undertaken in serum-free medium for HaCaT keratinocytes, while 1% FCS was used for both the normal dermal and the chronic wound-derived fibroblasts.

To investigate the effect of dextrin−rhEGF on proliferation, cells were seeded into 96-well microtiter plates (in the appropriate medium), at a cell density of $2.5 \times 10^3$ cells/well and incubated serum-free for 24 h. A stock solution of dextrin−rhEGF (50 μg/mL rhEGF-equiv in phosphate buffered saline PBS) was diluted to 6.4 μg/mL rhEGF-equiv with the appropriate medium used for each cell type and then incubated at 37 °C with or without α-amylase (93 IU/L) for 24 h. These samples were serially diluted and added to the cells (in triplicate) before incubation for 72 h when proliferation was assayed using the MTT dye-reduction assay.[29]

**Cell Migration Assay.** The HaCaT keratinocytes, normal dermal and chronic wound fibroblasts were seeded into 24-well culture plates (BD Biosciences, Oxford, U.K.) at a cell density of $2.5 \times 10^4$ cells/well in their respective culture medium (in this case with serum). Cells were incubated until 95−100% confluent and then serum-starved for 24 h. The cells were washed (×2) with PBS and samples added as follows (in the appropriate medium for each cell type): media alone, free rhEGF (6.25 ng/mL keratinocytes; 1.56 ng/mL fibroblasts), dextrin−rhEGF (0.1 ng/mL rhEGF-equiv keratinocytes; 0.02 ng/mL rhEGF-equiv fibroblasts) and dextrin−rhEGF (at the same concentrations) that had been incubated

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with α-amylase as described above. The concentrations used for each cell type were chosen based on the preliminary optimization studies reported in the Supporting Information). In studies with HaCaT keratinocytes, dextrin (1.56 $\mu$g/mL) and α-amylase (93 IU/L) controls were also set up. To start these migration studies, a single linear scratch wound was made centrally across each cell monolayer, using a pipet tip.[30] Cell migration was visualized over a 48 h period at 37 °C and 5% $CO_2$ using Automated Time-Lapse Microscopyphotography (Carl Zeiss, New York; using Openlab 5 Software, Improvision Ltd., Coventry, U.K.) in duplicate experiments. In the case of HaCaT keratinocytes, "wound closure" was quantified by image analysis (see Supporting Information for full details). In an attempt to quantify cellular migration of the chronic wound fibroblasts in response to the bioresponsive conjugates, cell migration, directionality and velocity were quantified using this system and compared to that observed in patient-matched normal fibroblasts.

**Data Analysis.** Statistical analyses were undertaken using GraphPad Prism Version 4.00 (GraphPad Software, San Diego, California). Data were compared using a Student's *t* test and a one-way ANOVA with a Bonferroni post-test, was used for group analysis. Results are expressed as a mean and standard deviation (SD) or standard error of the mean (SEM). All *P* values are two-tailed. Statistical significance was considered at a probability of $P < 0.05$.

## Results and Discussion

Due to the pressing clinical need for improved treatments for chronic wounds it was important to assess the practicality of the dextrin−rhEGF under conditions relevant to the likely clinical setting in order to judge potential before progression to *in vivo* evaluation. For this type of approach, a first clinical trial would probably entail localized, topical application of the conjugate in patients with diabetic or venous leg ulcers. As such, characterization of the wound environment was clearly important. The results of wound fluid analysis are summarized in Table 1. As α-amylase is essential for dextrin−rhEGF activation, it was important to confirm its presence in wound fluid samples. There was significantly greater α-amylase activity in the acute wound fluid (mean 188 ± 73 IU/L), compared to the chronic wound fluid (52 ± 5 IU/L), when expressed using standard IU/L (Table 1; Figure 1). Greater variation in α-amylase levels was noted in acute wound fluid (Table 1; Figure 1), but all samples measured fell within the normal human blood level range for α-amylase (23 to 85 IU/L; some laboratories give a range of 40 to 140 IU/L).[31] Given the harsh degradative environment, the lower α-amylase activity in chronic wound fluid

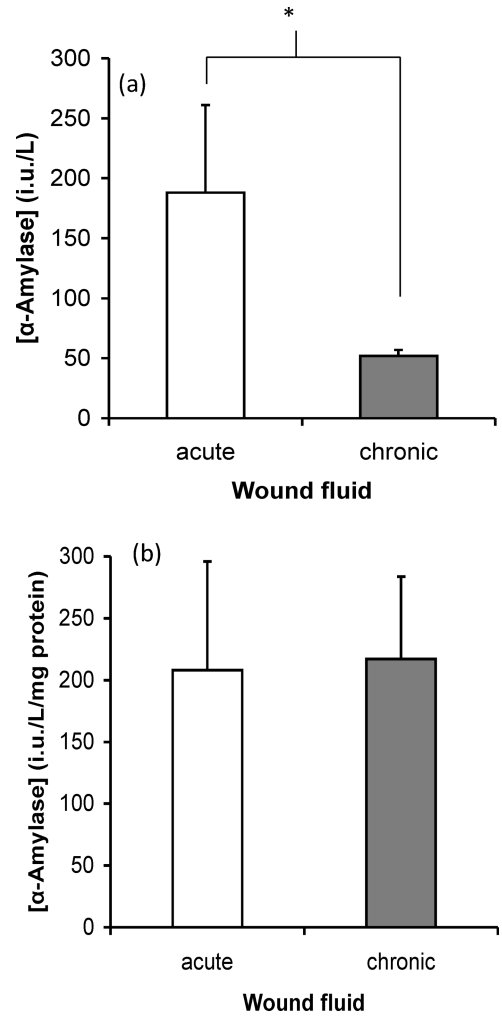


***Figure 1.*** Comparison of the levels of α-amylase in acute and chronic wound fluid. Panel (a) shows a significant difference between the α-amylase activity (expressed as IU/L) in acute and chronic wound fluid ($n = 5$; $^*P < 0.0001$). Panel (b) shows α-amylase activity corrected for protein content.

was not surprising, but as the total protein content of the acute wound fluid samples was also significantly higher compared to chronic wound fluid ($P < 0.0001$, $n = 5$), recalculation of α-amylase activity as a specific activity (with standardization mg of protein) in fact showed no significant difference between the levels of α-amylase in the acute and chronic wound fluid samples collected from these patients ($P > 0$ 0.05).

No elastase activity was detected in acute wound fluid, but it was present in chronic wound fluid (Table 1) where the mean activity was 2.1 ± 1.2 RFU/min, corresponding to 17 ± 8.8 RFU/min/mg protein. This equates to an elastase activity of 0.004 U/mL and an elastase concentration of 30 ng/mL. No EGF was detected in the chronic wound samples, but it was present in acute wound fluid (34 ± 22 pg/mL). This observation would be consistent with the EGF degradation by proteolysis and/or other factors in the aggressive chronic wound fluid environment. In support of this notion, addition of rhEGF to the chronic wound fluid sample taken

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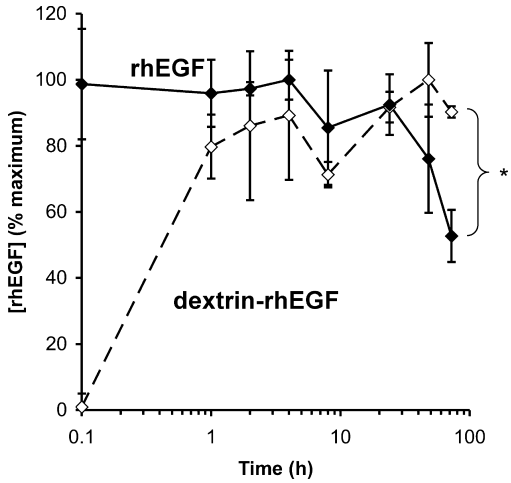


***Figure 2.*** rhEGF and dextrin−rhEGF stability in chronic wound fluid. The levels of rhEGF and dextrin−rhEGF measured are expressed as a percentage of the maximum concentration of rhEGF over time ($n = 3$, mean ± SD, $^*p = 0.0013$).

from patient C (with the highest elastase activity; Table 1) resulted in degradation over time with only 52% remaining at 72 h ($t_{1/2}$ ~76 h) (Figure 2). In contrast, when dextrin−rhEGF was added to this chronic wound fluid sample, at the outset no rhEGF was detected by ELISA assay (Figure 2). This observation was consistent with our previous studies[22] where α-amylase degradation of the dextrin−rhEGF conjugate was followed using FPLC to monitor the change in molecular weight (dextrin degradation and rhEGF release) and ELISA to follow the time-dependent release of immunoreactive rhEGF. These observations suggest that conjugated rhEGF is completely masked by the polymer that wraps around it. Here, using patient-derived chronic wound fluid, we showed that rhEGF would be liberated over time from the conjugate within the chronic wound environment (Figure 2). In this case dextrin is being degraded by endogenous wound fluid α-amylase. A maximal rhEGF concentration was achieved after ~48 h, and thereafter it appeared that rhEGF release from the conjugate and rhEGF removal by proteolytic degradation were in equilibrium. It should be noted that such ELISA experiments confirm only the liberation of immunoreactive protein. Further *in vivo* experiments will be needed to verify biological activity in the aggressive wound environment and also to define conjugate pharmacokinetic−pharmacodynamic relationships in that setting.

The main cell types involved in the structural processes associated with wound healing, i.e. collagen synthesis, ECM remodeling and re-epithelialization, are the fibroblasts and keratinocytes.[32] We have reported previously the significant differences between chronic wound fibroblasts and patient-matched, control normal dermal fibroblasts in terms of their significantly impaired wound healing phenotype, reduced proliferative life-span, early onset of senescence and decreased ability to withstand oxidative stress.[33,34] Fibroblasts derived from chronic wound tissue retain their wound phenotype, and although they migrate at a similar rate to normal dermal fibroblasts, they are slower to proliferate.[33] Importantly though, they maintain an ability to respond to rhEGF. Our preliminary studies showed that dextrin−rhEGF-induced proliferation of the high EGFR-expressing HEp2 cell line, and HaCaT keratinocytes,[22] but it was considered important here to investigate whether dextrin−rhEGF could influence the behavior of the phenotypically distinct chronic wound fibroblasts, i.e. the target cell. Time-lapse analyses of wound repopulation showed that both normal and patient-matched chronic wound fibroblasts were able to repopulate the *in vitro* scratch wound model (Figure 3a,b). Individual cell-tracking studies (Figure 3c) showed no significant difference in the response observed for normal and chronic wound fibroblasts ($p > 0.05$). While addition of the dextrin−rhEGF conjugate alone (in the absence of α-amylase) had no additional effect, exposure of the fibroblasts to free rhEGF or the dextrin−rhEGF conjugate preactivated with α-amylase significantly stimulated cell migration ($p < 0.05$; Figures 3d,e). Although the magnitude of stimulation was similar in both cases, it should be remembered that EGF was absent in the patient chronic wound fluid samples.

It is known that wound repopulation (in such *in vitro* models as well as *in vivo*) occurs as a result of both cellular migration and proliferation. Therefore fibroblast proliferation was also studied here. Both the normal and chronic wound fibroblasts displayed growth stimulation in the presence of free rhEGF (Figure 4). The response was biphasic with greatest stimulation at lower concentrations. Addition of α-amylase-activated dextrin−rhEGF also stimulated cell growth, producing a maximum effect that was similar to the maximum achieved by free rhEGF (normal dermal fibroblasts ~160% and chronic wound fibroblasts ~140%). Interestingly, for both normal and wound fibroblasts, the maximum effect produced by the conjugate was observed at a significantly lower rhEGF-equiv concentration (6−20 pg/mL) than required to elicit the same effect using native rhEGF (Figure 4), likely to be attributable to differences in the pharmacokinetic/pharmacodynamic (PK/PD) relationships of the protein and the conjugate. It should be noted that addition of α-amylase, dextrin alone or the succinoylated dextrin used for conjugation, did not influence cell proliferation in the HEp2 cell model.[22] The importance of the slow, sustained introduction of bioactive rhEGF, is underlined by its biphasic,

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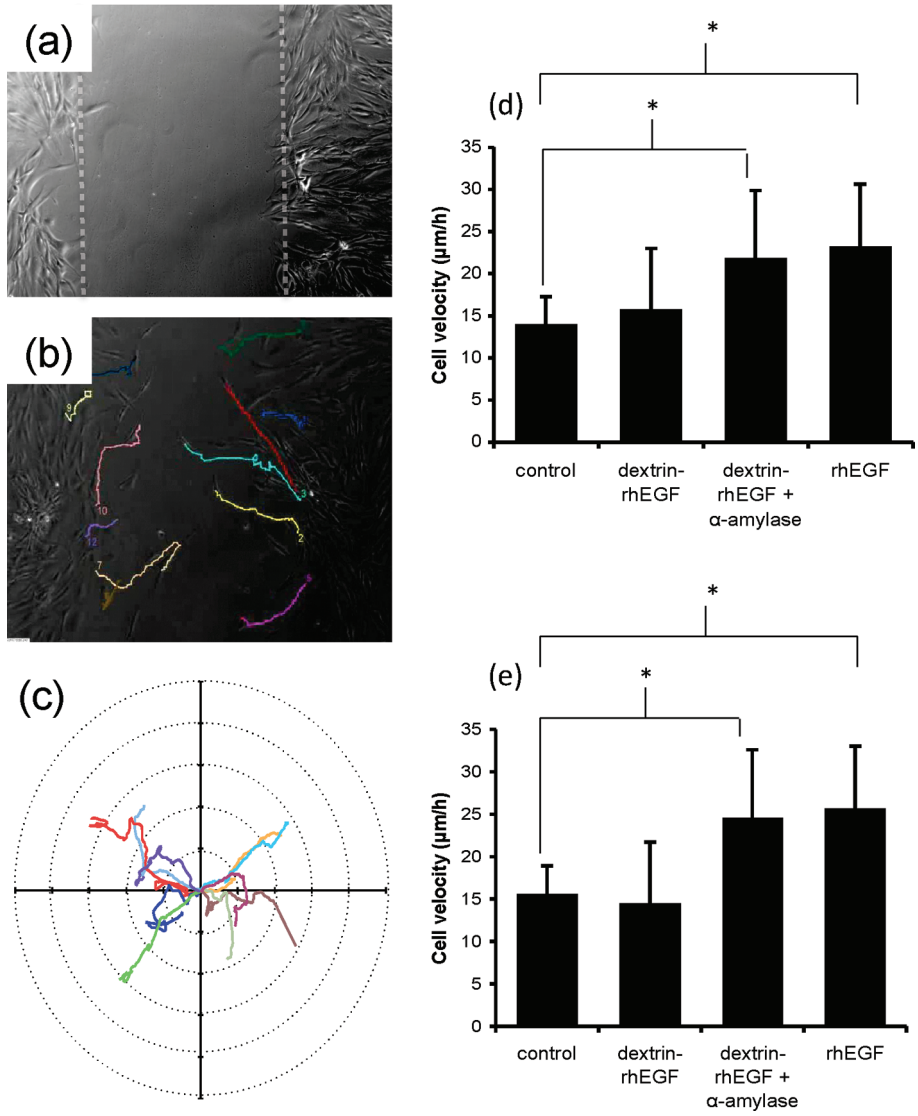


***Figure 3.*** Normal dermal and chronic wound fibroblast migration. The "scratch wound" data from typical fibroblast tracking experiments, are shown in panels (a)−(c). In each experiment, 10 typical fibroblasts were tracked over a 48 h period. Panels (d) normal dermal fibroblasts and (e) chronic wound fibroblasts show the migration velocity observed for individual cells, in response to dextrin−rhEGF (0.1 ng/mL equiv), dextrin−rhEGF + α-amylase (93 IU/L) and rhEGF (3.125 ng/mL). Data represent mean ± SD, $n = 10$, $^*p = 0.01$ (panel d) and $^*p < 0.0012$ (panel e).

concentration-dependent biological activity (Figure 4). Clearly, high local concentrations must be avoided if the inhibition of wound healing is to be averted.[35,36]

*In vivo*, wound closure by keratinocytes migrating from the surrounding epidermis is of pivotal importance. HaCaT keratinocyte migration was therefore also studied using the scratch wound assay. In this case the keratinocytes migrate to completely refill the wound space so the time-lapse wound closure data obtained could be analyzed digitally to quantify this "space-filling" response (Figure 5a and Supporting Information). Both rhEGF and the α-amylase-activated, dextrin−rhEGF conjugate significantly increased the rate and extent of "scratch closure" observed at 48 h ($P < 0.01$) (Figure 5b,d). Dextrin−rhEGF (without activation), α-amylase and succinoylated dextrin were unable to promote a similar response (Figure 5c). Again, clearly α-amylase is needed for dextrin−rhEGF to exert a biological response.

Currently, only one growth factor treatment is approved for use in chronic wounds. This is a topical application of recombinant PDGF (rhPDGF) in a carboxymethylcellulose gel (Regranex),[37] and it is licensed specifically for use in the treatment of diabetic foot ulcers. Whether or not it can achieve significant clinical benefit in all treated patients has

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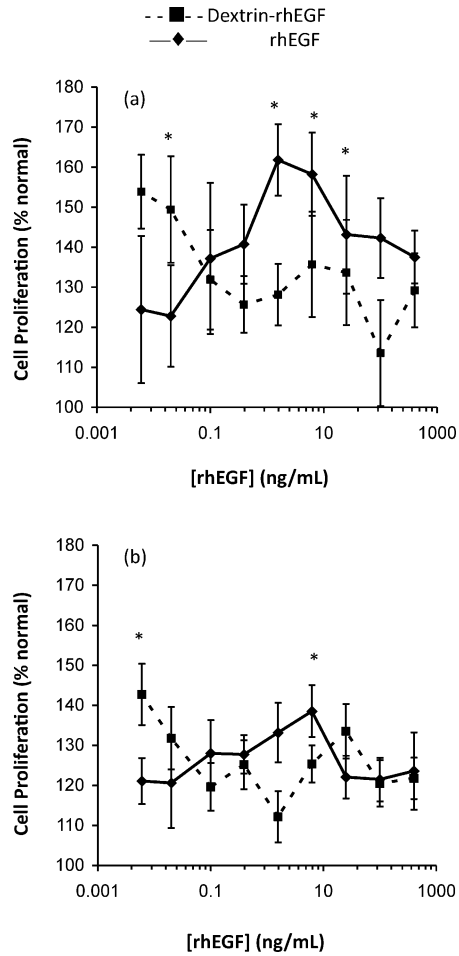


***Figure 4.*** Fibroblast proliferation response. Panel (a) normal dermal fibroblasts and panel (b) chronic wound fibroblasts, incubated with rhEGF or dextrin−rhEGF exposed α-amylase (93 IU/L), relative to the rhEGF-free control. Data represent mean ± SEM; $n = 3$; $^*P < 0.01$, by ANOVA and Bonferroni *post hoc* test.

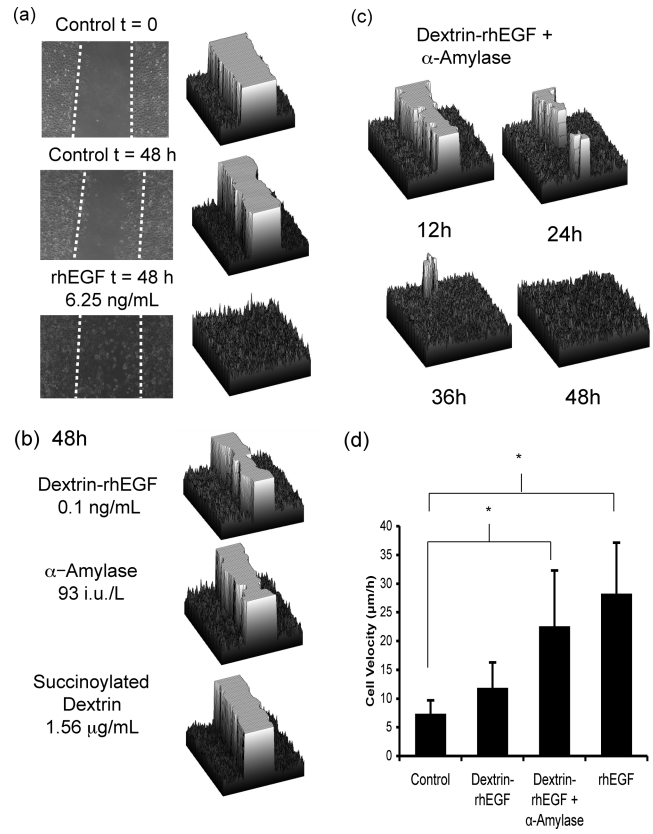


***Figure 5.*** HaCaT keratinocyte migration. Panels (a) and (b) show typical control data for this experiment, presented as either the visible "scratch wound" or the digital analysis of wound area (see Supporting Information). Panel (c) shows typical wound repopulation over time, on incubation with dextrin−rhEGF (0.1 ng/mL, following 24 h α-amylase exposure, 93 IU/L), and panel (d) shows estimated HaCaT cell migration velocity. Data represent mean ± SD, $n = 10$; $^*P = 0.0001$, by ANOVA and Bonferroni *post hoc* test.

been debated,[38] and last year concerns were raised that Regranex may have the potential to induce cancer and/or accelerate the progression of pre-existing malignant disease.[39] The fact that dextrin−rhEGF conjugates can be customized according to (i) the dextrin molecular weight[19] and (ii) the degree of dextrin succinoylation (which has been shown previously to control of the rate of polymer degradation by α-amylase,[26] and hence the bioactivation rate) provides an opportunity to fine-tune the design so that conjugation will protect the protein against the aggressive wound fluid environment, release bioactive protein locally at a controlled rate to meet pharmacological needs, and also enable optimization of the whole body pharmacokinetics to minimize exposure to non-target tissues. Of course future studies will be needed to precisely define the complex pharmacokinetic−pharmacodymamic relationship for dextrin−rhEGF, and also its metabolic fate in an *in vivo* setting, but the fact that conjugates optimized with respect to polymer molecular weight and degree of protein modification have already progressed into routine clinical use (e.g., those polyethyleneglycol (PEG)−protein conjugates reviewed in ref 40) suggests the practicality of this approach. There have been many reports of growth factor entrapment in carriers (e.g., liposomes, micro- and nanoparticles) and biodegradable scaffolds (reviewed in ref 41). As this novel approach proposes, for the first time, to use a bioresponsive polymer

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therapeutic, the concept could have much broader application to the delivery of other therapeutics to aid dermal and other tissue repair processes. It might also be optimized for various routes of application.

## Conclusions

Demonstration that α-amylase is present in the human wound fluid at levels able to release rhEGF from the dextrin−rhEGF conjugate, and that conjugate activated by incubation with α-amylase was able to stimulate *in vitro* proliferation and migration of normal dermal fibroblasts, chronic wound fibroblasts and keratinocytes, provides further evidence to support the viability of the PUMPT approach for use as a treatment for chronic wounds. Although growth factor depletion in the aggressive, chronic wound fluid environment (indeed no EGF was detected in the patient samples analyzed herein) has been implicated in impaired dermal wound healing, it must be remembered that growth factor replacement is only one aspect of this complex, multifactorial pathological process. Although it has been argued that there are no "perfect" animal models that correlate well with the human, chronic wound clinical situation, the results presented here have supported the commencement of *ex vivo* (rat corneal abrasion model) and *in vivo* (mouse diabetic wound healing model) studies to further ascertain preclinically, the therapeutic potential of the dextrin−rhEGF conjugate as a bioresponsive polymer therapeutic for chronic wound treatment.

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**Supporting Information Available:** Additional experimental details as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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