

Combined effects of PLGA and vascular endothelial growth factor promote the healing of non-diabetic and diabetic wounds

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Received 16 January 2015; accepted 8 July 2015

Abstract

Growth factor therapies to induce angiogenesis and thereby enhance the blood perfusion, hold tremendous potential to address the shortcomings of current impaired wound care modalities. Vascular endothelial growth factor stimulates (VEGF) wound healing *via* multiple mechanisms. Poly (lactic-*co*-glycolic acid) (PLGA) supplies lactate that accelerates neovascularization and promotes wound healing. Hence, we hypothesized that the administration of VEGF encapsulated in PLGA nanoparticles (PLGA-VEGF NP) would promote fast healing due to the sustained and combined effects of VEGF and lactate. In a splinted mouse full thickness excision model, compared with untreated, VEGF and PLGA NP, PLGA-VEGF NP treated wounds showed significant granulation tissue formation with higher collagen content, re-epithelialization and angiogenesis. The cellular and molecular studies revealed that PLGA-VEGF NP enhanced the proliferation and migration of keratinocytes and upregulated the expression of VEGFR2 at mRNA level. We demonstrated the combined effects of lactate and VEGF for active healing of non-diabetic and diabetic wounds.

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Key words: Diabetic wound healing; Poly(lactic-*co*-glycolic acid) (PLGA); VEGF; Lactate; Nanoparticles

Background

Dermal wound healing proceeds in a very systematic and step-wise manner beginning with hemostasis and inflammation followed by robust cellular proliferation, extracellular matrix (ECM) deposition, angiogenesis and remodeling, and ultimately scar formation. All these events happen in acute stages of healing.¹ Pathological conditions such as diabetes, arterial and venous insufficiency and lymphedema may lead to chronic wounds with compromised microcirculation. In these diseases, localized ischemia and edema inhibit the delivery of oxygen and nutrients to the tissues, impairing wound healing. Thus, chronic wounds fail to restore the anatomical and functional integrity of the skin and remain for longer periods.² So far more than 6.5 million patients are affected by impaired wounds in the USA alone. An estimated excess of US\$25 billion is spent annually on treatment of chronic wounds and the burden is rapidly growing.³ Diabetic wounds are one of the most prevalent impaired

Kiran Kumar Chereddy and Claudia Moia are early stage researchers (ESR) of FP7 Marie Curie NANODRUG network. Pierre Sonveaux and Anne des Rieux are Research Associates, Gaëlle Vandermeulen is a Postdoctoral Researcher and Valéry L Payen is a PhD fellow of the Belgian Fonds National de la Recherche Scientifique (F.R.S. — FNRS).

The authors declare no conflict of interests.

This research work is completely supported by the European Commission and Marie Curie Actions (grant number: PITN-GA-2011-289454).

This research work will be presented at Controlled Release Society Conference 2015, Edinburgh on July 27th, 2015.

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wounds in the world. In these wounds, growth factor levels have been proven to decrease and insufficient blood perfusion coupled with impaired angiogenesis complicates tissue repair, but for an efficient diabetic wound healing, a proper angiogenesis is pivotal.⁴ Growth factor therapies to induce angiogenesis and thereby enhance blood perfusion hold tremendous potential to address the shortcomings of current diabetic wound care modalities.^{5,6}

Among growth factor families, one of the most important proangiogenic mediators is the set of vascular endothelial growth factor (VEGF). Sufficient VEGF levels are believed to be essential for wound healing. VEGFa is a powerful therapeutic tool for proangiogenic therapy in many settings. VEGFa stimulates wound healing *via* multiple activities including deposition of collagen, angiogenesis and re-epithelialization.^{7,8} A large number of studies already reported that VEGF application *via* injection or topical routes enhances the wound healing.⁹ However, VEGF administration presents critical limitations due to its low *in vivo* stability and immediate degradation by proteases in the wound bed. Thus, treatment may require high dose and dosing frequency of VEGF¹⁰ or gene therapy¹¹ to produce the therapeutic effect, suggesting the need of efficient drug delivery systems for VEGF.

Poly(lactic-co-glycolic acid) (PLGA) is an attractive polymer because of its excellent biocompatibility, biodegradability, high safety profile, widespread use in medicine and is approved by Food and Drug Administration (FDA) and European Medical Agency (EMA) as an excipient in parenteral products. The release kinetics of this system can be easily adjusted by altering the ratio of poly(lactic acid) to poly(glycolic acid).¹² Recently, Porporato et al. reported that the exogenous application of lactate delivered from PLGA can accelerate wound healing. The published results also suggested that utilization of PLGA is one of the best strategies to supply lactate sustainably.¹³ Effects of lactate include promotion of angiogenesis, activation of procollagen factors and improved recruitment of endothelial progenitor cells in wounds.¹⁴ In our previous work, we have extensively studied the dual roles of PLGA in wound healing: a wound healing agent itself *via* lactate and it is able to hydrolytically degrade and release loaded drugs sustainably.^{15,16} Moreover, PLGA nanoparticles (NP) have been proved to be efficient carriers for VEGF and can accelerate angiogenesis.¹⁷

To our knowledge, PLGA and VEGF application in acute or diabetic wound healing and investigation of their combined effects have never been reported before. In this study, we hypothesized that VEGF administration in PLGA nanoparticles (PLGA-VEGF NP) could fasten the wound healing due to the combined effects of lactate and VEGF. A mouse full thickness excision model was used to evaluate PLGA-VEGF NP efficacy and to study their mechanisms of actions in both non-diabetic and diabetic wound healing.

Methods

VEGF₁₆₄ production and radio labeling

Recombinant VEGF₁₆₄ (VEGF hereafter) was produced and purified as previously described.¹⁸ The integrity of VEGF was

Table 1
Sequences of oligomers.

Oligo name	Sequence of primer (5' → 3')
VEGFR2 forward	GGAAGGCCCATTTAGTCCAA
VEGFR2 reverse	TTGGCGTAGACTGTGCATGT
β-Actin forward	TACAATGAGCTGCGTGTGGCC
β-Actin reverse	AGGATGGCGTGAGGGAGAGCAT

evaluated by SDS-PAGE and the concentration was determined by UV spectrophotometry (calculated dimer molar extinction coefficient: $\epsilon = 12,115 \text{ M}^{-1} \text{ cm}^{-1}$) and ELISA (using monoclonal anti-rat VEGF and biotinylated anti-rat VEGF (R&D Systems Inc., USA), Pierce® high sensitivity streptavidin–horseradish peroxidase and TMB substrate kit (Thermo Scientific, USA)). Pure VEGF ($98.9\% \pm 2.6$, ~40 kDa) was successfully collected. The obtained VEGF was aliquoted and stored at -80°C .

PLGA-VEGF nanoparticles preparation

VEGF was encapsulated in PLGA (50:50, MW 7000–17,000, acid terminated, Ingelheim GmbH, DE) using W/O/W emulsion–solvent evaporation technique with a few modifications from the literature.¹⁹ Detailed procedure was mentioned in the section of supplementary material.

PLGA-VEGF nanoparticles characterization

Nanoparticle size and poly dispersity index (PDI) were measured by dynamic light scattering and the zeta potential was determined using a zeta potential analyzer (NanoSizer Zeta Series, Malvern Instruments, UK).

Encapsulation efficiency and drug loading of PLGA-VEGF NP were estimated by quantifying the VEGF in supernatants. Lyophilized PLGA-VEGF NP (1 mg) was dissolved in 1 ml of chloroform and 1 ml of water to collect the encapsulated VEGF which was then estimated by ELISA.

Stability studies and drug release profile

A known quantity of VEGF was kept in PBS at room temperature and stability was assessed at different days (0, 2, 7 and 12) by ELISA. To study the stability of NP over time, 10 mg of lyophilized PLGA-VEGF NP was taken into 5 ml glass vials that were sealed with plastic caps and were kept in stability chamber with temperature of $37 \pm 2^\circ\text{C}$ and relative humidity $60 \pm 5\%$. On days 0, 10, 20 and 30, the NP were measured for particle size, PDI, zeta potential and drug loading as described in PLGA-VEGF nanoparticles characterization.²⁰

Four mg of PLGA-VEGF NP (containing 5.04 μg VEGF) was dispersed in 500 μl of PBS and pelleted by centrifugation at 12,000g for 10 min and placed at room temperature (RT). On several time points till day 30, 200 μl of supernatant was aspirated and stored at -80°C and the same amount fresh PBS was added to the solution.¹⁶ The amount of VEGF released was estimated as described in PLGA-VEGF nanoparticles characterization. The cumulative release of VEGF from the NP was calculated by dividing the cumulative VEGF released into the

Table 2
Properties of PLGA-VEGF NP and PLGA NP (mean \pm SD, n = 4).

Name	Particle size (nm)	Poly dispersity index	Zeta potential (mV)	Encapsulation efficiency (%)	Drug loading (μ g VEGF/mg PLGA NP)
PLGA-VEGF NP	203 \pm 9	0.07 \pm 0.02	-30 \pm 2.0	75.8 \pm 4.7	1.26 \pm 0.06
PLGA NP	163 \pm 2	0.15 \pm 0.05	-21 \pm 3.5	NA	NA

buffer by the total amount of VEGF incorporated inside the PLGA-VEGF NP.¹⁸

In vivo lactate release studies were performed to understand the influence of enzymes and other biofactors on release kinetics. 6-7 weeks old RjHan:NMRI female mice (Janvier, BE) were injected subcutaneously on the back with 100 μ l of Hanks' Balanced Salt Solution containing either no nanoparticles (untreated) or 10 mg of PLGA-VEGF NP. Dialysate was collected on anesthetized mice (ketamine/xylazine) right after injection on days 0, 7 and 14 using CMA/20 microdialysis probes with 4 mm long and 100 kDa cut-off membrane (Aurora Borealis, SE) implanted following manufacturer's instructions at the injection site. NaCl 0.9% was perfused through the CMA/400 Syringe Pump (Aurora Borealis, SE) at a rate of 1 μ l/min. After discarding 7 first μ l corresponding to the dead volume of the probe and outlet tubing, dialysate was harvested for 30 minutes. L-Lactate concentration in the harvested samples was determined by enzymatic-based dosage using a CMA/600 Microdialysis Analyser (Aurora Borealis, SE) following manufacturer's instruction.

In vivo wound healing assay

In vivo wound healing evaluation was performed following the literature.^{15,21} Briefly, 6-7 weeks old RjHan:NMRI (n = 40) and 7-8 weeks old *db/db* (n = 20) female mice (Janvier, BE) were randomly grouped. Leptin receptor deficient *db/db* mice were used for diabetic wound model. Mice were shaved on their dorsal area using a depilatory cream (Veet for sensitive skin, BE) a day before the surgery to avoid irritation/rashes. After anesthetizing, two (8 mm diameter) and four (6 mm diameter) full-thickness excisional wounds were made using round skin biopsy punch (Kai Europe GmbH, DE) on either side of the dorsal midline for NMRI and *db/db* mice respectively. 0.5 mm-Thick silicone (Grace Biolabs, UK) donut-shaped splints were fixed around the wounds and positioned with 6-0 nylon sutures (Monosof, USA). For NMRI mice, 10 mice/group were randomly chosen and wounds were treated intradermally at 4 sites around each wound with either only vehicle (0.9% w/v NaCl, Mini-Plasco, BE), 6 μ g VEGF, 5 mg PLGA NP or 5 mg PLGA-VEGF NP (equivalent to 5 mg PLGA and 6 μ g VEGF) in 30 μ l of vehicle by a sterile insulin syringe (BD Medical, FR). For *db/db* mice, wounds were treated with same doses as that of NMRI mice. Wounds on the same mouse received different types of treatments. Wounds were covered with bandages (IV3000, Smith & Nephew, UK) and at several time points, wounds were digitally photographed. Wound areas were quantified by using Jmicro Vision software (University of Geneva, CH) and were expressed as percentage of the respective initial wound area. 3 Mice/group were sacrificed on days 5 and 10 (NMRI) or days 9 and 18 (*db/db*) and wounds along with surrounding tissue were

collected for further experiments. Remaining animals were sacrificed at the end point of experiment which is complete wound healing. The animal studies were approved by the animal care and ethical committee, Université Catholique de Louvain (2014/UCL/MD/034).

Histology, Sircol collagen assay and immunohistochemistry

Wound tissue (n = 3) was immediately fixed with paraformaldehyde (4% in PBS, 0.01 M, pH 7.4) for 24 h and then transferred to PBS buffer at 4 °C. Wound tissues embedded in paraffin blocks were sectioned at 5 μ m using a MICROM 17M325 microtome (Thermo Fisher Scientific, DE). Sections were stained with hematoxylin and eosin (HE) and Masson's Trichrome (MT) green staining to study the morphology of skin layers and the extent of collagen deposition respectively.

Following the manufacturer's instructions, using Sircol collagen assay kit (Biocolor, UK) total acid-soluble collagen (types I-V) in the wound tissue samples (n = 3) (day 10 for non-diabetic and day 18 for diabetic) was quantified colorimetrically. Collagen concentrations were expressed as μ g collagen per gram of total protein.^{16,22}

Following the manufacturer's protocol CD34 immunohistochemical staining was performed to evaluate the presence of endothelial cells and extent of vessel formation in tissue sections (n = 3).²³ Briefly, sections were deparaffinized and rehydrated in 2-propanol. The endogenous peroxidase activity was blocked using H₂O₂ diluted in methanol. Sections were then incubated with goat serum for 30 min and then with rat anti-mouse CD34 antibody (1:100 dilution; HM1015, Hycult Biotech, NL) for 1 h. Endogenous biotin was blocked using a blocking kit (SP2001, Vector Laboratories, USA). Sections were incubated with rabbit anti-rat IgG biotinylated antibody (1:100 dilution; BA 4001, Vector Laboratories) for 1 h followed by streptavidin (1:500 dilution; P0397, Dako, BE) for 1 h. Diaminobenzidine served as a chromogen (K3468, Dako, BE). Slides were counterstained with HE and mounted with DPX neutral mounting medium (Sigma-Aldrich, DE).

Slides were scanned using Leica SCN400 slider scanner (Leica Biosystems, DE) and analyzed with Leica slide viewer software version 4.0.5. FRIDA software (Johns Hopkins University, USA) was used to semi-quantify the MT green staining and the staining values were expressed as staining per unit area. For CD34+ staining quantification, all vessels and endothelial cells present in wound area were defined and counted and the total vessel surface area was calculated.

Myeloperoxidase (MPO) assay

Azurophilic granules of neutrophils contain MPO and can be used as a quantitative index of inflammatory cellular infiltration.²⁴ Wound tissues from each group (n = 3) on days

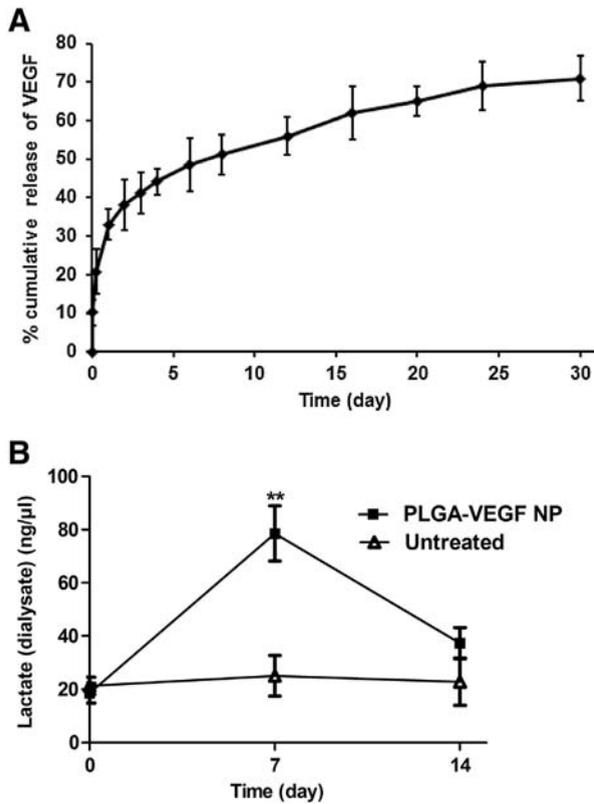


Figure 1. Release studies of PLGA-VEGF NP. (A) *In vitro* release profile of VEGF (mean \pm SD, $n = 3$) and (B) *In vivo* evaluation of lactate release (mean \pm SD, $n = 6$).

5, 10 (non-diabetic) and days 9, 18 (diabetic) were collected and myeloperoxidase assay was performed as described in the section b of supplementary material.

Quantification of VEGFR2 by q-PCR

From the wound tissue digestion total RNA was isolated using TRIzol[®] reagent (Ambion, Invitrogen, BE). Extracted RNA was treated with DNase I (Promega, USA) to remove any genomic DNA fragments and then quantified by nanospectrophotometer (NanoDrop 2000, Thermo Scientific, DE). The final concentration of RNA was adjusted to 1 $\mu\text{g}/3 \mu\text{l}$. Following the supplier protocol, 0.75 μg RNA was reverse transcribed using first standard synthesis system (SuperScript[™], Invitrogen, BE) and oligo (dT) primer (Eurogentec, BE) and stored at $-20 \text{ }^\circ\text{C}$. PerlPrimer software was used to design the desired primers based on murine mRNA sequences (Table 1). To amplify the cDNA, SYBR green real-time q-PCR (GoTaq q-PCR MasterMix kit, Promega, A6000) were conducted with primers using StepOne Plus Real-Time PCR System (Applied Biosystems, BE). β -Actin (housekeeping gene) and a blank were included in the measurements. Melting curves were analyzed for each run to assess the presence of unspecific PCR products. StepOne Software V2.1 was used to analyze results. The mRNA expression of VEGFR2 genes was calculated relative to the expression of corresponding β -actin, according to the delta-delta Ct method.

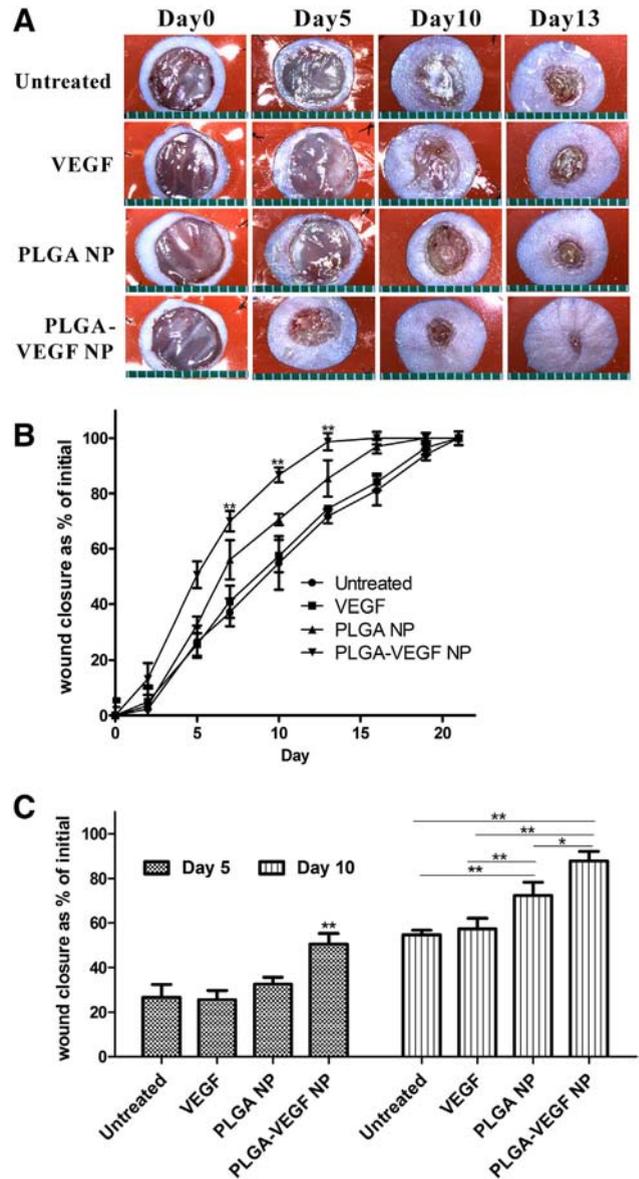


Figure 2. PLGA-VEGF NP accelerated wound healing in non-diabetic mice. (A) Representative images of wounds of the tested groups: Untreated, VEGF, PLGA NP and PLGA-VEGF NP. Ruler units in mm. (B) Quantitative representation of wound areas as function of time. (C) Wound area at day 5 ($n = 20$) and day 10 ($n = 14$) (mean \pm SD). Statistical significance compared with untreated group.

Proliferation, migration and cytotoxicity assay

HaCaT (ECACC, UK) keratinocytes and BJ fibroblast cells (ATCC 2522, UK) were cultured in an atmosphere of 5% $\text{CO}_2/95\%$ air (v/v) at $37 \text{ }^\circ\text{C}$ and in DMEM/F-12 with L-glutamine and 15 mM Hepes, supplemented with 10% (v/v) FBS (Invitrogen, UK).

Proliferation, migration and cytotoxicity assays were performed using HaCaT keratinocytes and BJ fibroblasts as described in the sections c, d, and e of supplementary material.

Statistical analysis

The data are presented as mean \pm SD. Statistical analysis was performed by using one-way ANOVA with the Tukey's test

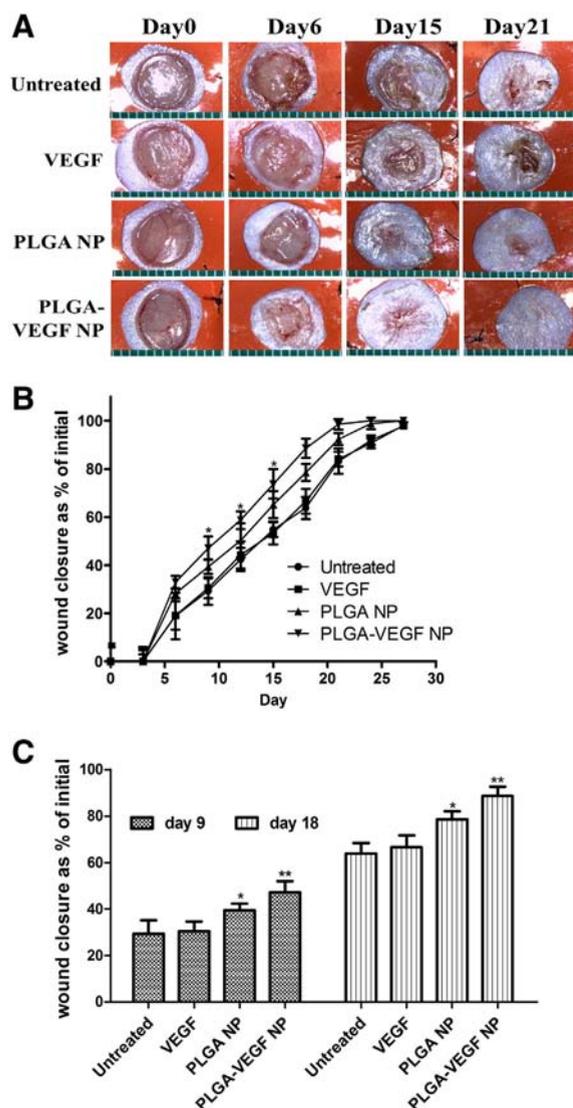


Figure 3. Wound healing effects of PLGA-VEGF NP on diabetic wounds. (A) Representative images of wounds of four tested groups: Untreated, VEGF, PLGA NP and PLGA-VEGF NP. Ruler units in mm. (B) Quantitative representation of wound areas as function of time. (C) Wound area at day 9 ($n = 10$) and day 18 ($n = 7$) (mean \pm SD). Statistical significance compared with untreated group.

applied post hoc for paired comparisons of means (GraphPad Prism 5.0). Values of $*P < 0.05$ and $**P < 0.01$ were indicative of statistically significant differences.

Results

Formulation, physico-chemical characterization, release and stability studies of PLGA-VEGF NP

VEGF was encapsulated in PLGA NP using double (W/O/W) emulsion–solvent evaporation technique with PVA as stabilizer. Table 2 presents the physico-chemical characterization (size,

PDI, zeta potential and encapsulation efficiency) of PLGA-VEGF NP.

VEGF at room temperature was found to be stable for about 12 days. The loss of VEGF was found to be $3.0 \pm 0.8\%$, $5.0 \pm 1.2\%$ and 7.8 ± 2.2 at days 2, 7 and 10 respectively. The stability studies of PLGA-VEGF NP for 30 days at room temperature revealed that there were no significant differences in properties (size, zeta potential and drug loading) and NP were stable throughout the studies (data not shown).

In vitro drug release profile of VEGF from PLGA-VEGF NP is shown in Figure 1, A. The release curve clearly exhibited a biphasic pattern with an initial burst release followed by a gradual and sustained release of VEGF. The burst release within 1 day resulted in more than 30% VEGF release into the supernatant and nearly 70–75% of VEGF released by day 30 suggesting that there was a sustained release. *In vivo* release studies revealed that lactate was released from PLGA, with a peak corresponding to $78.6 \text{ ng}/\mu\text{l}$ (concentration in the dialysates) at day 7 post NP implantation, followed by decrease back to baseline concentration (Figure 1, B).

PLGA-VEGF NP accelerated dermal wound healing

Using full thickness skin excision model, wound healing effects of PLGA-VEGF NP were evaluated in both non-diabetic and diabetic mice. Untreated (vehicle), VEGF ($6 \mu\text{g}$), PLGA NP (5 mg) served as controls. Compared to all the other groups, wounds treated with PLGA-VEGF NP showed a significantly faster wound closure both in non-diabetic (Figure 2, A, B) and in diabetic (Figure 3, A, B) mice. In case of non-diabetic mice, from day 5 PLGA-VEGF NP exhibited higher healing and by day 13 wounds were completely healed. Figure 2, C shows the healing % of different treatments at day 10: PLGA-VEGF NP 87% versus PLGA NP 72% versus VEGF 57% versus untreated 54%. PLGA NP treated group also followed the same pattern as PLGA-VEGF NP but at a slower rate. Untreated and VEGF groups showed much lower healing of wounds. In case of diabetic mice, wound healing showed slower kinetics but the response pattern was similar to that of non-diabetic wound healing. The acceleration of wound healing by PLGA-VEGF NP became significant from day 9, and the wounds were completely healed by day 19, showing an average healing % of $47 \pm 4.8\%$ and $98 \pm 2.0\%$, respectively (Figure 3, C). PLGA NP treated wounds also showed a considerably higher healing rate compared to the untreated and VEGF groups. Wounds that were either untreated or treated with VEGF alone showed poor healing at day 18 (untreated 64% and VEGF 66%) (Figure 3, C), and wounds were completely closed after 28 days. Although PLGA-VEGF NP showed significant wound healing activity and faster healing rate in both diabetic and non-diabetic wounds, its effect was much evident in the latter condition.

PLGA-VEGF NP promoted re-epithelialization, granulation tissue formation and neovascularization

Wound sections were stained with HE and MT to investigate the changes in morphology of skin layers and collagen extent during the healing process. For a clear visibility of morphology changes, day 10 and day 18 sections of non-diabetic and diabetic

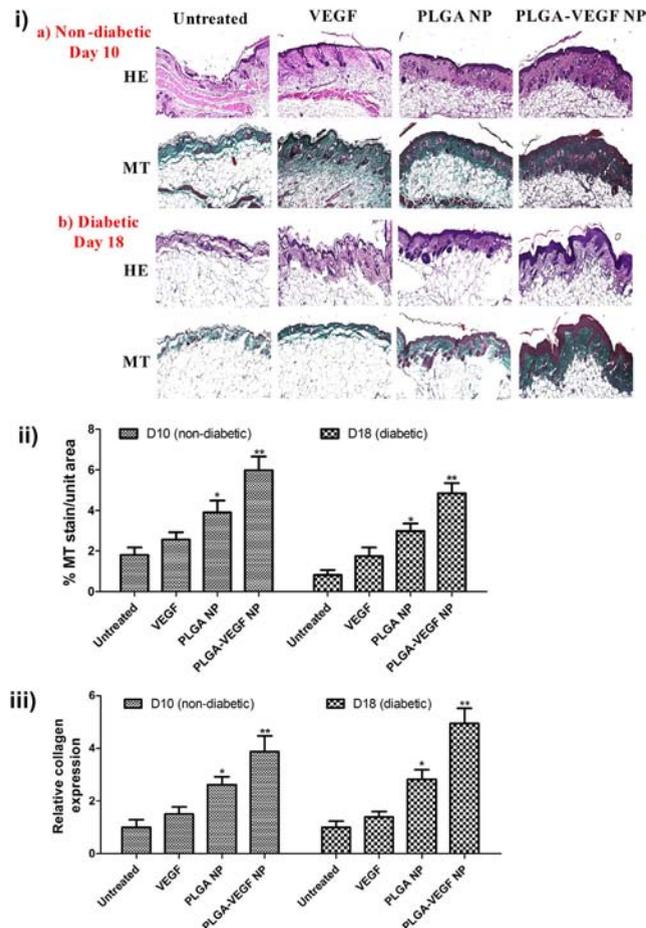


Figure 4. PLGA-VEGF NP treated wounds showed increased wound re-epithelialization and collagen content of granulation tissue. Both (ia) non-diabetic and (ib) diabetic wound sections were stained with hematoxylin and eosin (HE) and Masson's Trichrome (MT) (collagen in green). Representative images are presented. Scale bar = 100 μ m, n = 3. (ii) Semi-quantitative estimation of MT staining of wound sections. Results are expressed as MT stain per unit area (n = 3). (iii) Colorimetric analysis of acid-soluble collagen in wound lysates (n = 3).

wounds were presented respectively. In both non-diabetic (Figure 4, ia) and diabetic (Figure 4, ib) wounds treated with PLGA-VEGF NP showed well organized skin layers. The arrangement of epidermal and sub-epidermal layers was tight and matured. Although untreated, VEGF and PLGA NP treated wounds displayed various degrees of migration of epithelium over dermis, only untreated and VEGF treated wounds showed poor arrangement of dermal layers and hypo-cellular epithelialization. The extent of collagen deposition as well as its alignment at the healing area was measured by MT staining (Figure 4, ii) and Sircol collagen assay (Figure 4, iii). Wounds treated with PLGA-VEGF NP showed a greater granulation tissue formation, collagen deposition and dermal remodeling compared to the other groups. PLGA NP treated wounds also showed a well aligned and dense collagen pattern compared to PLGA-VEGF NP treated wounds, whereas untreated and VEGF treated wounds exhibited a loose and poor reticular collagen deposition.

CD34 immunolabeling of endothelial cells revealed that PLGA-VEGF NP treatment significantly promoted angiogenesis

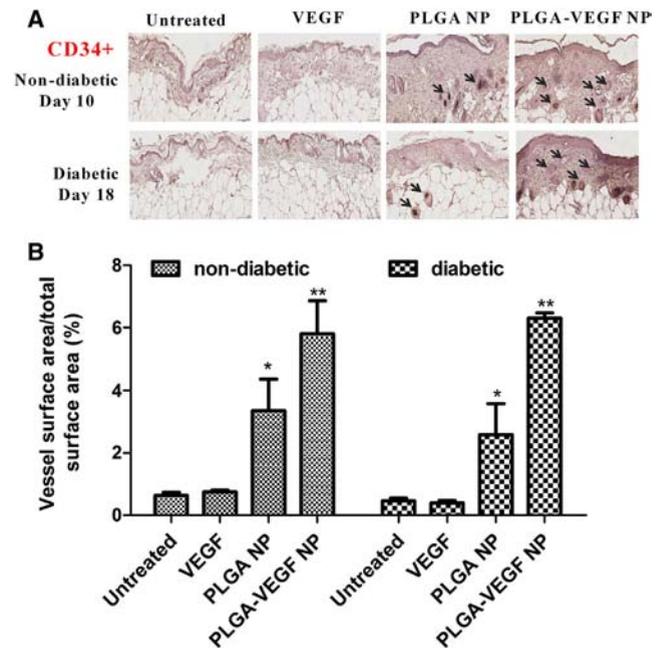


Figure 5. PLGA-VEGF NP enhanced the angiogenesis. Sections of non-diabetic and diabetic wounds were stained with CD34 immunohistochemistry staining. (A) Representative pictures of IHC staining of CD34+ marking (scale bar = 100 μ m, arrows indicate infiltrating blood capillaries), (B) quantification of vessel area representing the extent of CD34 marking (mean \pm SD, n = 3). Statistical significance compared with untreated group.

and blood capillary infiltration in wound sections (Figure 5, A). On days 10 (non-diabetic) and 18 (diabetic) post wounding, the mean staining area of CD34+ cells was quantified (Figure 5, B).

PLGA-VEGF NP showed no effect on myeloperoxidase activity

MPO units were found in higher amounts in diabetic wound tissues than that of the non-diabetic. PLGA-VEGF NP did not show any effect on MPO activity. From day 5 to day 10 (non-diabetic) (Figure 6, A) and day 9 to day 18 (diabetic) (Figure 6, B), MPO units were reduced in all the groups.

PLGA-VEGF NP regulated the expression of VEGFR2 at mRNA level

VEGFR2 plays an important role in angiogenesis and collagen deposition processes of wound healing.⁷ The effects of lactate and VEGF on the expression VEGFR2 at mRNA level in the wound tissues of untreated and groups treated with VEGF, PLGA NP and PLGA-VEGF NP were evaluated by q-PCR. It was measured at late stages of wound healing. Figure 6, C shows that PLGA formulations significantly ($P < 0.01$) upregulated the expression of VEGFR2. VEGF treated group showed the same level of expression as that of untreated group.

Effects of PLGA-VEGF NP on HaCaT keratinocytes and BJ fibroblasts

HaCaT keratinocytes and BJ fibroblast cell cultures were used to test the effects of PLGA-VEGF NP on their proliferation and metabolic activity. The Quant-iT result after 4 days of cells incubation with different formulations (500 ng/ml VEGF,

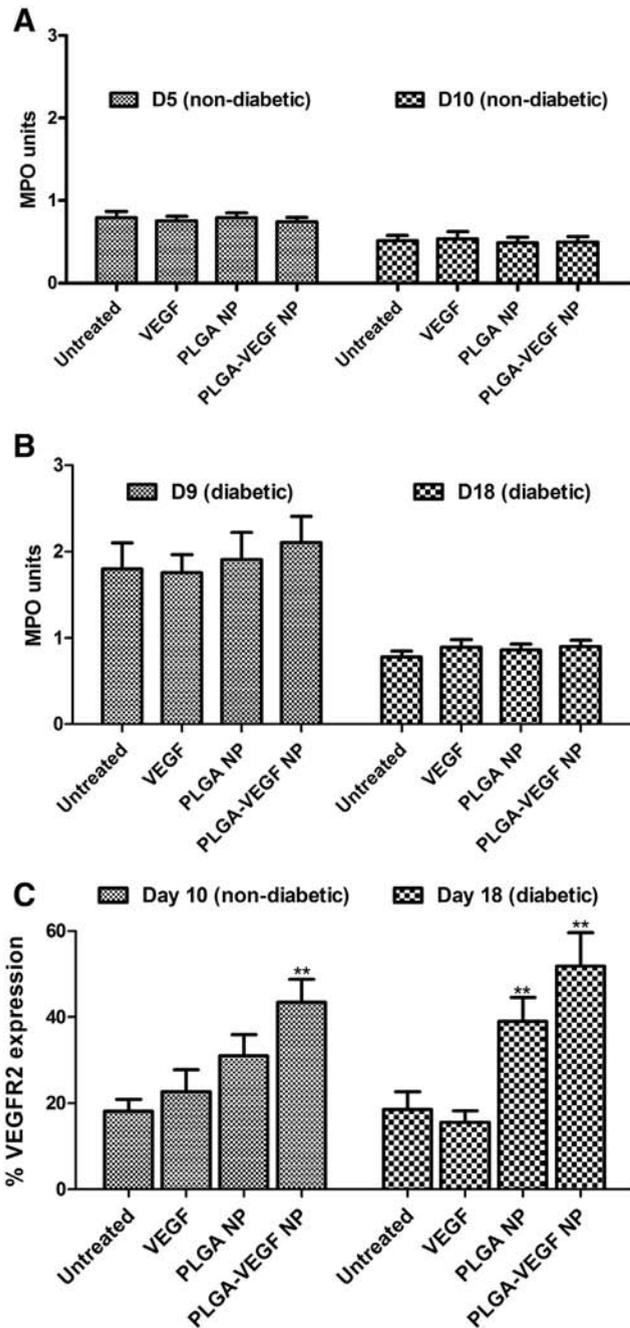


Figure 6. Wound tissue analysis to quantify MPO and VEGFR2 expression. MPO activity in different wounded tissues on (A) days 5, 10 (non-diabetic) and (B) days 9, 18 (diabetic) of post wounding (mean \pm SD, $n = 3$). (C) Relative mRNA levels of VEGFR2 were measured by q-PCR. Result was expressed as percentage of untreated. (mean \pm SD, $n = 3$).

500 μ g/ml PLGA NP and 500 μ g/ml PLGA-VEGF NP) clearly showed that the VEGF and PLGA-VEGF NP significantly promoted the proliferation of HaCaT keratinocytes whereas there was no influence on mitotic activity of BJ fibroblasts (Figure 7).

After 24 h, HaCaT cells and BJ fibroblasts treated with either free VEGF or PLGA-VEGF NP showed faster migration than the other groups. Free VEGF showed quick migratory effect whereas PLGA-VEGF NP showed significant effect from 12 h

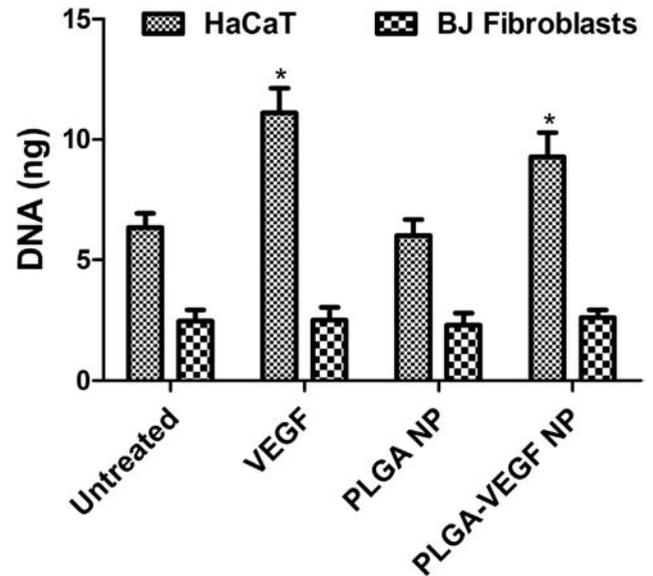


Figure 7. Effects of PLGA-VEGF NP on proliferation of dermal cells. DNA quantification of HaCaT keratinocytes and BJ fibroblasts was carried out after 4 day incubation with formulations ($n = 5$, mean \pm SD). Statistical significance compared with untreated group. Statistical significance compared with untreated group.

post wounding (Figure 8). As the migration assay was for 24 h, minimum effect of proliferation was observed.

MTT and LDH assays revealed that at the tested concentration, no formulation showed a significant effect on cell viability and mortality (data not shown).

Discussion

Growth factors and other biomolecules play important roles in wound healing.⁸ Research has been focused not only on how to use growth factors for the treatment of impaired wounds but also on how to deliver these growth factors efficiently without losing their bioactivity. There are significant efforts that mainly deal with developing new drug delivery systems to release growth factors in a controlled manner. One among those attempts is our PLGA based nanoformulations where the combined effects of lactate from PLGA hydrolysis and loaded VEGF were used for the treatment of diabetic wounds.

Purified VEGF was used to produce PLGA-VEGF NP (~75% of encapsulation efficiency and ~200 nm size) by W/O/W double emulsion–solvent evaporation method. Although the double emulsion technique yields high loading, it has several drawbacks such as in-process protein degradation in organic phase and different parameters to be considered (duration of sonication and centrifugation, possible interactions between polymer/stabilizer and protein), which may influence the encapsulation efficiency.²⁵ These might be the reasons why there was only 75% encapsulation efficiency and about 30–40% polymer loss in our case. *In vitro* release studies presented the overall idea of the order of kinetics. Lactate release studies were performed as a part of our previous studies that showed that there was a sustained release of lactate from PLGA degradation.¹⁶

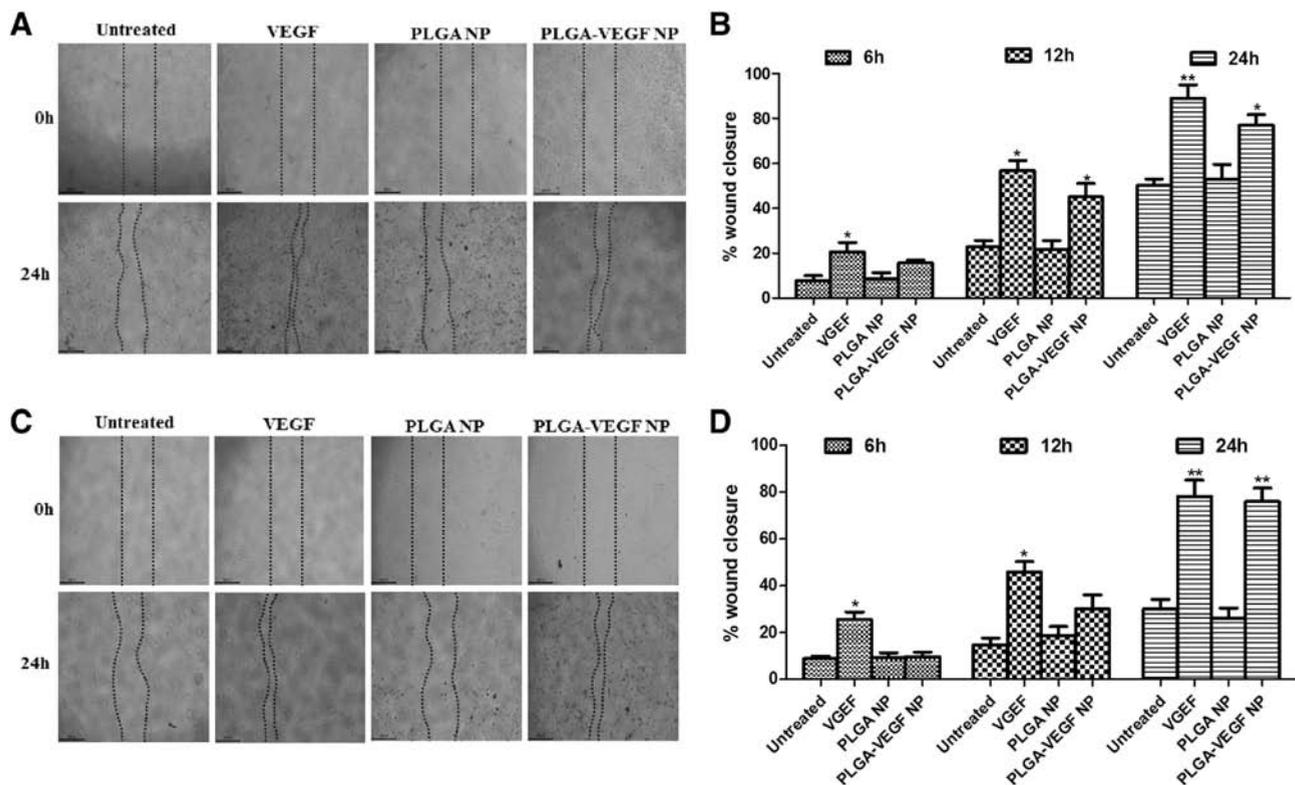


Figure 8. *In vitro* wound closure effects of PLGA-VEGF NP. Effect of VEGF, PLGA NP and PLGA-VEGF NP on migration of (A) HaCaT cells and (C) BJ fibroblasts was assessed by IBIDI wound healing assay. Wound closure of (B) HaCaT cells and (D) BJ fibroblasts were expressed as percentage of initial wound (mean \pm SD; n = 4). Statistical significance compared with untreated group.

VEGF release from PLGA-VEGF NP followed a biphasic pattern (initial burst release followed by sustained release) which is primarily due to degradation of NP by water absorption, pore formation leading to hydrolysis of intra-polymer linkages, auto-catalysis and erosion.²⁶ The release kinetics could much vary in the *in vivo* environment due to the presence of enzymes in wounds. In order to estimate *in vivo* release kinetics, microdialysis coupled to the enzymatic quantification of lactate was used in mice subcutaneously implanted with 10 mg of PLGA-VEGF NP. Release of lactate indicates that PLGA-VEGF NP can be exploited for sustained lactate delivery and the result in commitment with the literature.¹³ The *in vivo* release studies of lactate highlighted that there was a great influence of enzymes and other biofactors on the degradation of PLGA polymer.

Wound healing effects of PLGA-VEGF NP were evaluated in a splinted mouse full thickness excisional model. Silicone splints avoid the contraction of wounds and thereby the wound healing is mainly by re-epithelialization similar to that of humans. The inclusion of controls (untreated, VEGF and PLGA NP) presents insights into the individual abilities and activities in wound healing and their comparative validation. From our previous works^{15,16} and considering the molecular weight of PLGA polymer, 5 mg/wound dose was chosen for PLGA-VEGF NP and the controls were determined accordingly. The healing kinetics of both non-diabetic and diabetic wounds clearly showed that PLGA-VEGF NP treated mice exhibited a

significant faster wound closure than the other groups. The healing effect was significant from day 5 and day 10 for non-diabetic and diabetic wounds respectively. Within a few days, PLGA-VEGF NP induced nearly complete wound closure. It is worth mentioning that PLGA NP showed faster wound healing than the other controls. Thus, the significant wound healing activities of PLGA-VEGF NP and PLGA NP were in support of hypothesis of combined effects of lactate and encapsulated VEGF.

We studied the progress of wound healing, morphological changes in the skin layers, degree of re-epithelialization and extent of collagen deposition by histological examination of HE and MT stained sections prepared from all the groups. PLGA-VEGF NP treated wounds showed well developed and organized epithelium. The epidermal layers were tight and well matured. The wound architecture was almost re-built in case of both non-diabetic and diabetic wounds treated with PLGA-VEGF NP. MT stained sections and Sircol assay clearly showed that PLGA-VEGF NP enhanced granulation tissue formation and synthesis of a compact and denser collagen alignment. These histology observations prompted us to explore the effects of PLGA-VEGF NP at cellular and molecular level. To establish whether PLGA-VEGF NP influences keratinocytes and fibroblasts, we tested the formulation on HaCaT keratinocytes and BJ fibroblasts. The proliferation effect of PLGA-VEGF NP along with the controls was evaluated by incubating them with cells for

4 days. Although there was no proliferation effect on BJ fibroblasts, PLGA-VEGF NP and VEGF showed a significant mitosis induction effect on HaCaT keratinocytes. The effect was due to VEGF, which may alter the survival, proliferation, or stemness of keratinocytes in an autocrine manner.²⁷ Moreover, the fact that keratinocytes express functional VEGFRs implies that VEGF can directly influence these cells during wound repair.²⁸ Better re-epithelialization with PLGA-VEGF NP was also attributed to the effect of VEGF on keratinocyte and fibroblast migration that was evident from the *in vitro* wound healing assay. VEGF is a strong chemo attractant for keratinocytes and fibroblasts that promotes their migration and recruitment into the active mitotic region of wounds.²⁹ Lactate and hypoxic conditions may also contribute to the migration and proliferation of dermal cells at the wound site.⁷ Thus, the active proliferation and migration effects of VEGF and PLGA contributed to the active re-epithelialization and granulation tissue formation.

VEGF acts through tyrosine kinase receptors VEGFR1, VEGFR2, and VEGFR3 (predominantly expressed in vascular endothelial cells) mediated pathways. Of these, VEGFR2 is expressed primarily in the blood vascular endothelium and is considered to be the main mediator of angiogenesis and vascular permeability.³⁰ Upon binding of VEGF, phosphorylation of tyrosine residues on VEGFR stimulates activation of protein kinase B, which inhibits apoptosis, and of the mitogen-activated protein kinase (MAPK) pathway, which induces cell proliferation.³¹ Thus, VEGFR2 play an important role in wound healing particularly in angiogenesis.⁷ Hence to understand the mechanisms and effects of lactate and VEGF at molecular level, VEGFR2 expression was quantified by q-PCR. PLGA-VEGF NP significantly upregulated VEGFR2 mRNA expression. Kumar et al. reported that HUVECs (human umbilical vein endothelial cells) responded to high levels of lactate by upregulating VEGF and its endothelial-specific receptor, VEGFR2 which further modulated the angiogenic potency of VEGF through poly-ADP ribosylation (PAR)-dependent mechanism and thereby the switch over to angiogenic phenotype.³² VEGF acts not only in a paracrine manner to attract and stimulate the proliferation of endothelial cells, but can also act in an autocrine manner, having a positive/survival effect on a number of cell types including endothelial cells, embryonic stem cells and hematopoietic stem cells; resulting in further production of VEGF and expression of VEGFR2.³³ PLGA-VEGF NP treated wounds showed significantly higher CD34+ staining of endothelial cells. All these results collectively demonstrate that lactate and VEGF released from PLGA-VEGF NP induced significant neovascularization.

PLGA-VEGF NP showed no effect on neutrophil infiltration and myeloperoxidase release. Using most conventional methods, we have performed cytotoxicity assays on HaCaT and fibroblast cells at the given concentration of nanoparticles. PLGA-VEGF NP did not induce any cytotoxic effect on HaCaT keratinocytes and BJ fibroblasts. PLGA is a biocompatible and biodegradability polymer with wide applications in medicine and is approved by FDA and EMA as an excipient. VEGF is biomolecule produced and purified in our laboratory. Therefore, provided that our PLGA-VEGF NP were produced under sterile

conditions, the translation from animal to patient could be easily achieved and the approval from an ethical committee for clinical trial should be accepted without any further toxicity testing.

Thus, the combined effects of lactate (PLGA) and VEGF accelerated wound closure through different mechanisms of action targeting different cells that are actively involved in wound healing. Hence, the discussed results justify the use of PLGA and VEGF combination in impaired wound healing.

Acknowledgments

We express our sincere thanks to Mr. Bernard Ucar, Mr. Kevin Vanvarenberg and Ms. Aurélie Staub (ADDDB, Louvain Drug Research Institute, UCL, BE) for their kind help in animal experiments and tissue analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2015.07.006>.

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