Carbohydrate Polymers 147 (2016) 251–260

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Epidermal growth factor loaded heparin-based hydrogel sheet for skin wound healing

MeeiChyn Goh[a,1], Youngmin Hwang[a,1], Giyoong Tae[a,b,*]

[a] School of Materials Science and Engineering, Gwangju Institute of Science and Technology, 123 Cheomdan-gwagiro, Buk-gu, Gwangju 61005, Republic of Korea

[b] Center for theragnosis, Biomedical research institute, KIST, Seoul 02792, Republic of Korea

ARTICLE INFO

Article history:
Received 2 February 2016
Received in revised form 22 March 2016
Accepted 23 March 2016
Available online 31 March 2016

Keywords:
Heparin
Hydrogel
Epidermal growth factor
Wound healing
Skin

ABSTRACT

A heparin-based hydrogel sheet composed of thiolated heparin and diacrylated poly (ethylene glycol) was prepared *via* photo polymerization and human epidermal growth factor (hEGF) were loaded into it for the purpose of wound healing. It showed a sustained release profile of hEGF *in vitro*. In order to evaluate its function on wound healing *in vivo*, full thickness wounds were created on the dorsal surface of mice. Application of hEGF loaded heparin-based hydrogel sheet accelerated the wound closure compared to the non-treated control group, hEGF solution, and hEGF loaded PEG hydrogel sheet. Histological and immunohistological examinations also demonstrated an advanced granulation tissue formation, capillary formation, and epithelialization in wounds treated by hEGF loaded heparin-based hydrogel compared to other groups, and no biocompatibility issue was observed. In conclusion, the delivery of hEGF using the heparin-based hydrogel could accelerate the skin wound healing process.

1. Introduction

Cutaneous wound healing is a complex process involving several overlapping stages that include hemostasis, inflammation, proliferation, and wound remodeling with a series of well-ordered biological process (Strodtbeck, 2001). Wound healing requires the cellular interactions between several cell types such as keratinocytes, fibroblast, endothelial cells, neutrophils, and macrophages with the mediation of endogenously released growth factors, cytokines and chemokines at the wound site (Gailit & Clark, 1994). To assist the healing process, exogenous growth factors can be employed, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and transforming growth factor beta (TGF-β) families (Gailit & Clark, 1994; Bennett & Schultz, 1993). Among them, epidermal growth factor (EGF), a single polypeptide chain of 53 amino acids with three interchain disulfide bonds required for biological activity (Laato, Niinikoski, Lebel, & Gerdin, 1986), plays an essential role in accelerating and enhancing wound healing by stimulating proliferation of most epithelial cells, fibroblasts, endothelial cells, and even collagen deposition (Gainza et al., 2015) (Chen et al., 1993). It also stimulates the migration and proliferation of keratinocyte (Ando & Jensen, 1993).

An ideal wound dressing should maintain a moist environment around the wound that encourages re-epithelialization process (Winter, 1962), absorb the excess exudates from the wound bed, allow gaseous fluid exchange, and protect wound from bacterial infection (Yang et al., 2010; Yang, Yang, Lin, Wu, & Chen, 2008). Furthermore, it should facilitate absorption of wound caring growth factors by increasing the retention as well as their bioactivities at the wound site. Moreover, wound caring growth factor containing wound dressing should be non-adherent to the wound and ease to apply and remove without trauma (Sudheesh Kumar et al., 2012). For this purpose, hydrogels are attractive due to their potential to offer an ideal condition for wound healing. Due to their high intrinsic water content as well as swelling ability to absorb large amount of exudates from the wound bed, they can provide a moist environment to the wound, thereby protecting the wound from dehydration (Ajji, Mirjalili, Alkhatab, & Dada, 2008). Besides, hydrogels can facilitate the transport of water vapor and oxygen (Vernon, 2000). Furthermore, they are non-particulate, non-toxic, and non-adherent (Thomas & Hay, 1995). Numerous polymeric hydrogels composed of natural and synthetic polymers have been used to deliver wound caring growth factors to the wound site (Choi & Yoo,

* Corresponding author at: School of Materials Science and Engineering, Gwangju Institute of Science and Technology, 123 Cheomdan-gwagiro, Buk-gu, Gwangju 61005, Republic of Korea.
E-mail addresses: chyn87fish@gist.ac.kr (M. Goh), ymhwang@gist.ac.kr (Y. Hwang), gytae@gist.ac.kr (G. Tae).

[1] Both authors contributed equally to the work as joint first authors.

http://dx.doi.org/10.1016/j.carbpol.2016.03.072
0144-8617/

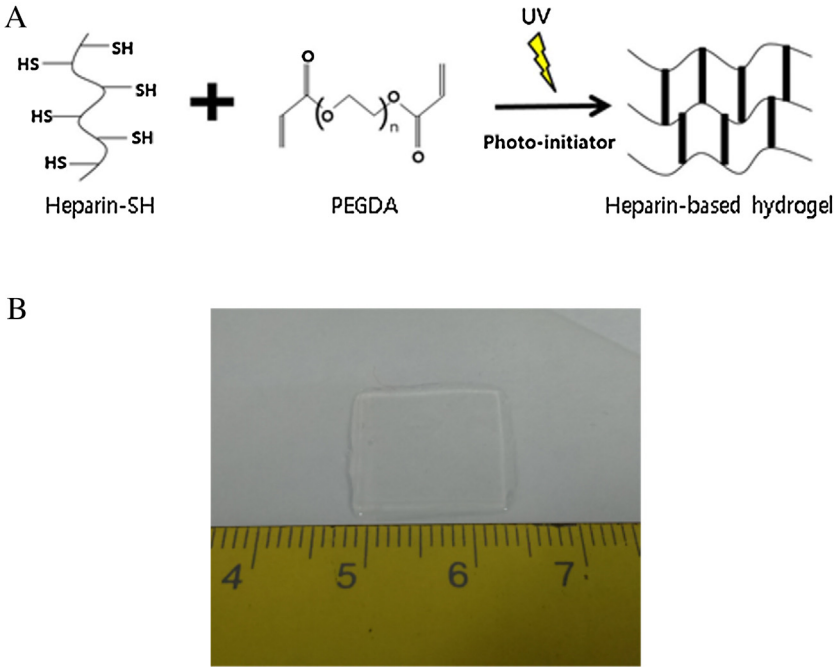

Fig. 1. (A). Schematic diagram of preparing heparin-based hydrogel sheets by photo polymerization. (B) Photographic appearance of the heparin-based hydrogel sheet.

Table 1
Gel content, swelling ratio, and storage modulus of hydrogel sheets (mean ± std, n = 4).

Sample	Molar Ratio(thiol: acryl)	Gel (%)	Swelling Ratio	Storage modulus before swelling (kPa)	Storage modulus after swelling (kPa)
Heparin-based hydrogel sheet	1:1	97.9 ± 0.7	14.4 ± 0.2	13.8 ± 1.0	10.6 ± 0.8
PEG hydrogel sheet	1:1	96.2 ± 1.5	14.9 ± 0.5	13.6 ± 0.5	9.4 ± 0.5

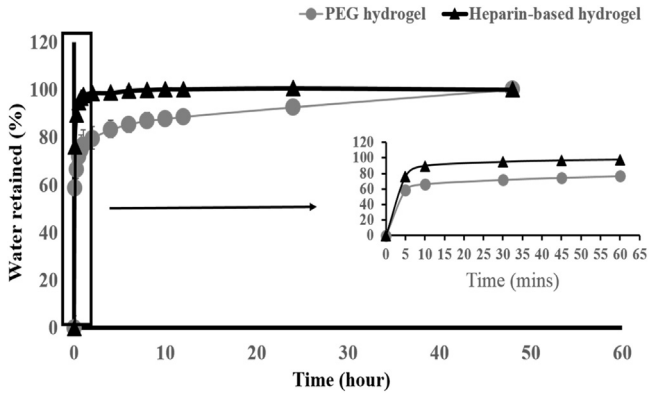

Fig. 2. Swelling behaviors of hydrogel sheets in PBS at 37° C.

2010; Lin et al., 2015; Hori et al., 2007). Those studies showed the acceleration of the wound site recovery by using designed hydrogel containing wound caring growth factor. A sustained released of wound caring growth factor from the hydrogels to overcome the unstable, short half-life of growth factors *in vivo* (Chen & Mooney, 2003), was one of the key parameters for the wound healing efficacy.

Heparin is a highly sulfated natural glycosaminoglycan that has high and specific affinities to various growth factors (Bennett & Schultz, 1993). Binding growth factors to heparin can extend their half-lives by protection from proteolytic degradation, and increase their bioactivity (Schultz & Wysocki, 2009). Johnson and Wang had reported controlled delivery of heparin-binding EGF-like growth factors yields fast and comprehensive wound healing by a bioactive heparin-based coacervate (Johnson & Wang, 2013). In our previous studies, we prepared bioactive heparin-based hydrogel systems, which showed the sustained release of human growth hormone (Choi, Kim, Tae, & Kim, 2008a) and hepatocyte growth factor (Shah, Kim, Cahill-Thompson, Tae, & Revzin, 2011), enhanced proliferation of chondrocyte (Kim, Shin et al., 2010), hepatocyte (Kim, Lee, Jones, Revzin, & Tae, 2010) and mesenchymal stem cells (Kim, Kim, & Tae, 2013) as well as cartilage regeneration (Kim, Kim, Kang, Kim, & Tae, 2011). In the present study, we prepared epidermal growth factor loaded heparin-based hydrogel sheet *via* UV-photopolymerization. The efficacy of the prepared hydrogel on skin wound healing was characterized both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

Heparin sodium (12 kDa, from porcine intestinal mucosa) was purchased from Cellus, Inc. (Cincinnati, OH, USA). Potassium chloride, potassium phosphate monobasic, sodium chloride, sodium phosphate dibasic, ethylcarbodiimide hydrochloride (EDC), 1-hydroxy-benzotriazole hydrate (HOBt), cysteamine hydrochloride, DL-dithiotreitol (DTT), chloramine T trihydrate, 30% *p*-dimethylaminobenzaldehyde, *n*-propanol and perchloric acid were purchased from Sigma (St. Louis, MO, USA). 4-(2-Hydroxyethoxy) phenyl-(2-hydroxy-2-propyl) ketone (Irgacure2959) was purchased from Aldrich (Milwaukee, WI, USA). 5,5′-Dithio-bis-(2-nitrobenzoic acid) (DTNB) was purchased from Pierce (Rockford, IL, USA). Poly (ethylene glycol) diacrylated (PEG-DA, degree of substitution: 98%, 3.4 kDa and 6 kDa), and poly (ethylene glycol) sulfurhydryl (PEG-SH4, 10 kDa) were purchased from Sunbio Inc. (Anyang, Korea). Human Epidermal Growth Factor (hEGF) was pur-

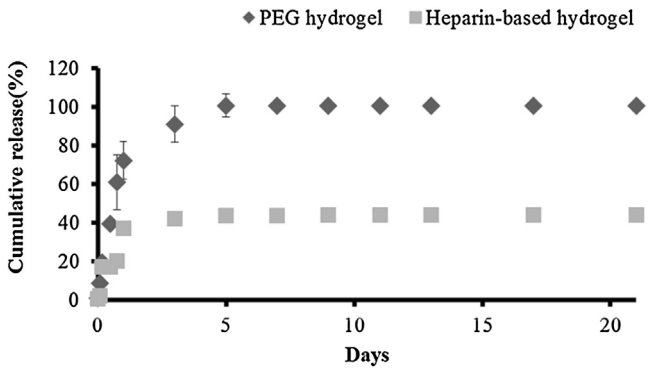

Fig. 3. Release of hEGF from hydrogel sheets in PBS with sodium azide (0.0065 wt%) at 37° C.

chased from Cha Biotech, (Seoul, Korea). All materials were used without further purification.

2.2. Preparation of photo crosslinked heparin-based hydrogel sheet

Thiolated-functionalized heparin (Hep-SH) was synthesized by converting 40% of carboxylic groups in 12 kDa heparin to thiol groups as previously reported (Tae et al., 2007). Photopolymerization of heparin-based hydrogel within a min had been previously demonstrated using Irgacure 2959 as photoinitiator with 365 nm, 18 W cm^{-2} UV light (Kim et al., 2013; Shah et al., 2011). For preparing heparin-based hydrogel sheet, 40% thiolated heparin and 6 kDa PEG-DA with 1:1 molar ratio of thiol group and acrylate group were dissolved in PBS containing 0.1 (w/v) photo-initiator in 70% v/v ethanol, and then exposed to 365 nm, 18 W cm^{-2} UV light using an OmniCure series 1000 light source (EXFO, Vanier, Quebec, Canada) with 3 cm distance between the sample and the UV source for 20 s. PEG hydrogel sheet, prepared by 10 kDa tetrafunctional PEG-SH and 3.4 kDa PEG-DA with 1:1 molar ratio of thiol group and acrylate group, was used as a control group. The final concentration of the gel precursor solution was 10% w/v in all cases.

2.3. Gel content

After photopolymerization, hydrogel sheets were first lyophilized for 2 days until a constant weight (W_d) was reached. They were next extracted in 37° C PBS for 2 days to remove the soluble part. The swollen hydrogel sheets were then lyophilized again for 2 days (W_g). The gel content (%) was defined a $\left(W_g/W_d\right) \times 100$.(1)

2.4. FT-IR analysis

FTIR spectra were obtained using an FT-IR spectrometer (Perkin Elmer Spectrum 2000, Waltham, MA, USA). FTIR samples were prepared by KBr pastille method. The lyophilized hydrogel samples were ground with KBr powder and the mixture was pelletized. The spectra were recorded in the range 400–4000 cm^{-1} with a resolution of 4 cm^{-1}. The scan number was 32

2.5. Swelling behavior

Prepared hydrogel sheets were extracted in 37° C PBS for 2 days. Fully swollen hydrogel sheets were then weighed (W_s) immediately after removing excess PBS on the surface by dabbing gently with wet Kimwipe. The swollen hydrogel sheets were lyophilized again for 2 days to a constant weight (W_d). The swelling ratio was calculated as W_s/W_d (2) (Fu, Gwon, Kim, Tae, & Kornfield, 2015).

The swelling kinetics of hydrogel sheets were measured by extracting dried hydrogel sheets (W_d) in 37° C PBS. At designed time points, excess PBS on the surface of hydrogel sheets were removed by using wet Kimwipe and weighed (W_t) until they reached the equilibrium state of swelling (W_s).The swelling kinetics of hydrogel sheets were calculated by the following equation (Yang, Liu, Chen, Yu, & Zhu, 2008).

$$\text{Water retained}\,(\%) = \frac{W_t - W_d}{W_s - W_d} \times 100. \quad (3)$$

2.6. Rheological measurement

The rheological measurements were conducted using a Rheometer (Gemini, Malvern Instruments, UK) equipped with a temperature controller at 37 °C and a solvent trap to suppress the drying of prepared hydrogel sheet. Hydrogel sheets after photopolymerization and after swelling in 37 °C PBS for 2 days were analyzed with a sandblast parallel plate geometry. Hydrogel sheets before and after swelling were cut in 15 mm diameter to fit the sample holder geometry (n = 4). All rheological measurements were carried out at frequency sweep from 0.1 to 100 rad s^{-1} with 0.1% strain (Kim et al., 2013).

2.7. Water vapor transmission rate (WVTR)

The moisture permeability of the hydrogel sheets were determined by measuring its water vapor transmission rate. A cuvette with an exposure area 1 cm^2 was filled with 3 mL deionized water and covered with PDMS mold where swollen hydrogel sheet had been previously attached on it (n = 4). The cuvette was placed in a 37 °C incubator. The weight of the cuvette was measured every hour and a graph of water evaporation *vs.* time was plotted. Measurements taken at least seven points gave a straight line ($R^2 \geq 0.99$). The slope of the curve was calculated and the WVTR value was evaluated using the following equation (Peles & Zilberman, 2012):

$$WVTR = \frac{Slope \times 24}{area}\left[\frac{g}{m^2 \times day}\right]. \quad (4)$$

2.8. In vitro *human epidermal growth factor (hEGF) release from hydrogel sheet*

hEGF was loaded into hydrogel by soaking 10 μL of 100 ng human epidermal growth factor in PBS to a 1 cm^2 hydrogel sheet with 1 mm thickness at 4° C for 1 day to induce complete sorption. Before starting the release experiment, a 12 well polystyrene plate was pre-coated with 0.1% BSA to prevent nonspecific adsorption sites in order to minimize the loss of released growth factor. A release medium, 800 μL PBS with 1 mM sodium azide, was added to the wells containing hEGF loaded hydrogel. The samples were then kept at 37° C in a humidified 5% CO_2 incubator. The release media were collected at the designed time points and replaced with the fresh one. Collected release media were frozen immediately prior to analysis. The amounts of released hEGF at different time points were analyzed by a hEGF ELISA kit using 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonicacid (ABTS), Sigma, St. Louis, MO, USA) as a substrate, and the absorbance was measured at 405 nm using a microplate reader (n = 4).

2.9. Cutaneous wound healing model

Balb/c mice (8-week-old, male, Orient Bio, Iksan, Korea) were housed under standard conditions of controld temperature and humidity (25 °C ± 1, 40%) and a light/dark cycle (12/12 h). One day before the wounding, the dorsal hair of mice were shaved with an electric shaver and completely removed by "Veet" cream (Oxy

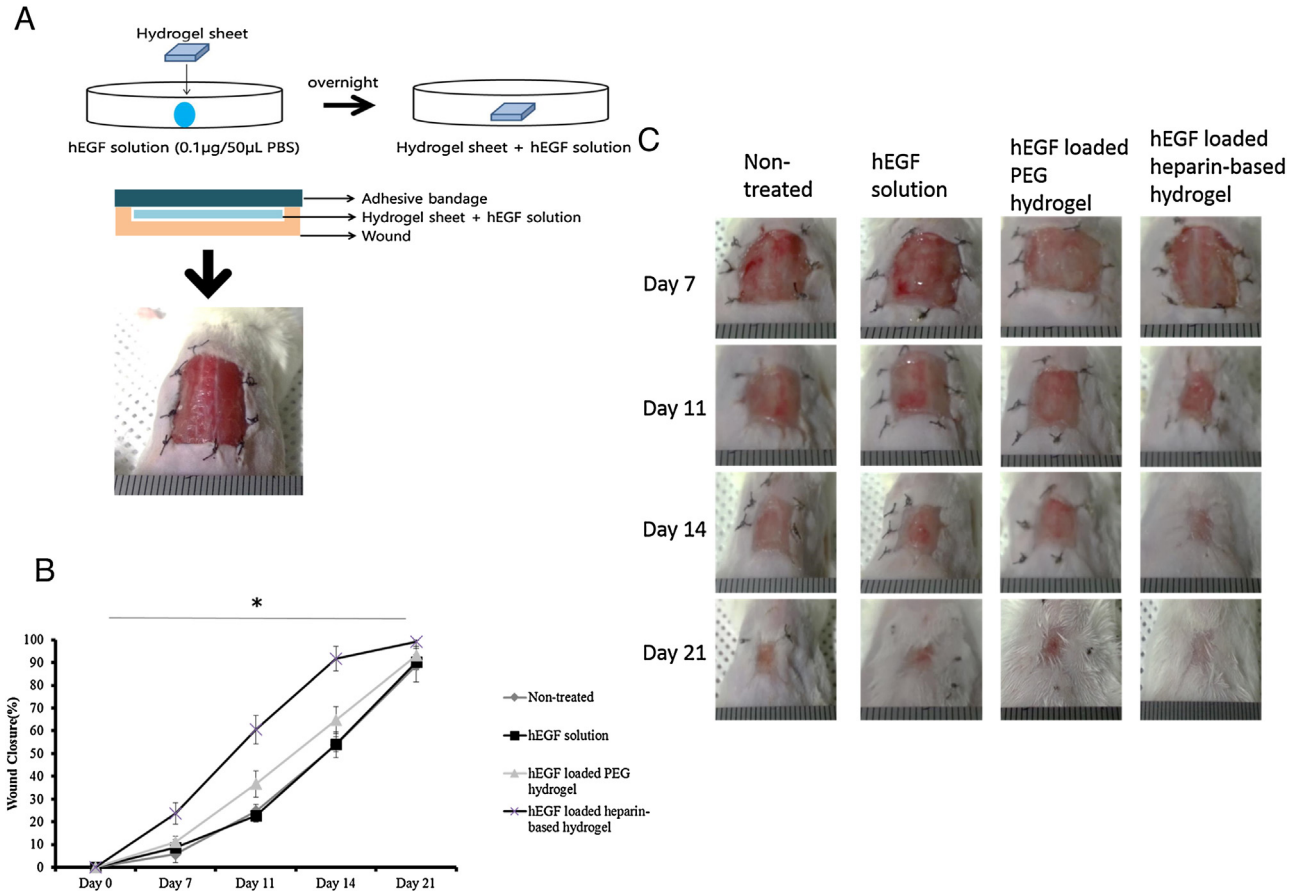

Fig. 4. (A) Experimental scheme of preparing hEGF loaded hydrogel sheet and wound healing treatment for full thickness wound. (B) Wound closure over time in wound healing model. *: $p < 0.05$. (C) Photographs of macroscopic appearance of wounds.

Reckitt Benckiser, Seoul, Korea). On the day of wounding, mice were anesthetized with isoflurane (JW Phamarceutical, Seoul, Korea). A full thickness skin wound of 1.5 cm^2 was prepared by excising the dorsum of mice using surgical scissors and forceps. Eight sutures were made at the edge of wounds with 5-0 suture (Ailee, Busan, Korea) to prevent the contraction of the wound, and the border of wound was connected with the muscle fascia. The wounded mice were randomly distributed into 4 groups with six per group (n = 6) and received treatment; (1) control group (non- treated), (2) EGF group (only hEGF solution, 100 ng/50uL), (3) PEG group (hEGF loaded PEG hydrogel) and (4) Heparin group (hEGF loaded heparin-based hydrogel). The dimension of the hydrogel sheets were 1.5 cm^2 with 1 mm thickness. After treatment, all wounds of mice were covered with an adhesive bandage (Sinsin Pharm, Ansan, Korea). At various time points (0, 7, 11, 14, and 21 days), the wound area was measured by using a transparent film and a pen (Sudheesh Kumar et al., 2012). The wound closure was calculated.

$$\text{Wound closure}\,(\%) = \frac{Area\ on\ day\ 0 - Area\ on\ day\ n}{Area\ on\ day\ 0} \times 100\,(\%) \qquad (5)$$

On day 21, mice were sacrificed and the skin wounds tissue were collected for histological and immunohistological evaluation. Animal experiments were carried out following the ethical regulations of Gwangju Institute of Science and Technology, Gwangju, Korea.

2.10. Histological examination

The skin wound tissue was excised using surgical scissors and fixed in a 10% formalin solution. The wound tissue was then dehydrated in different grades of ethanol and xylene and embedded in paraffin to prepare tissue sections. Wound tissue was sectioned in 5 μm thick layer by using a manual microtome (RM2235, Leica, Wetzlar, Germany). After deparaffinized and rehydrated, the tissue sections were proceeded by H&E staining for morphological analysis and by Sirius Red staining for collagen staining as well as the collagen distribution at the wound site. Stained sections were visualized and imaged by using optical microscopy (TE2000-U, Nikon Co, Tokyo, Japan).

2.11. Collagen quantification- hydroxyproline assay

Hydroxyproline assay was carried out to determine the total amount of collagen in the regenerated skin, following the published protocols with some modification (Lai, Lim, & Kim, 2011). Briefly, the regenerated skin samples were collected and 6 N hydrochloric acid was added to the samples. The samples were kept at 80° C for 24 h, followed by neutralization with NaOH in a vacuum oven at 80° C to obtain dry samples. After that, 100 μL chloramines T reagent (10 μL 0.6 M chloramine T trihydrate in deionized water, 90 μL mixture of isopropyl alcohol and acetate-citrate buffer (1:1 volume ratio)) was treated to the dried samples for 10 min at room temperature. Then, Ehrlich's reagent (30% *p*-dimethylaminobenzaldehyde in DMSO, *n*-propanol/perchloric acid mixture (2:1) with volume ratio 1:1) was added and incubated for 90 min at 60° C. Absorbance was measured at 550 nm with a microplate reader.

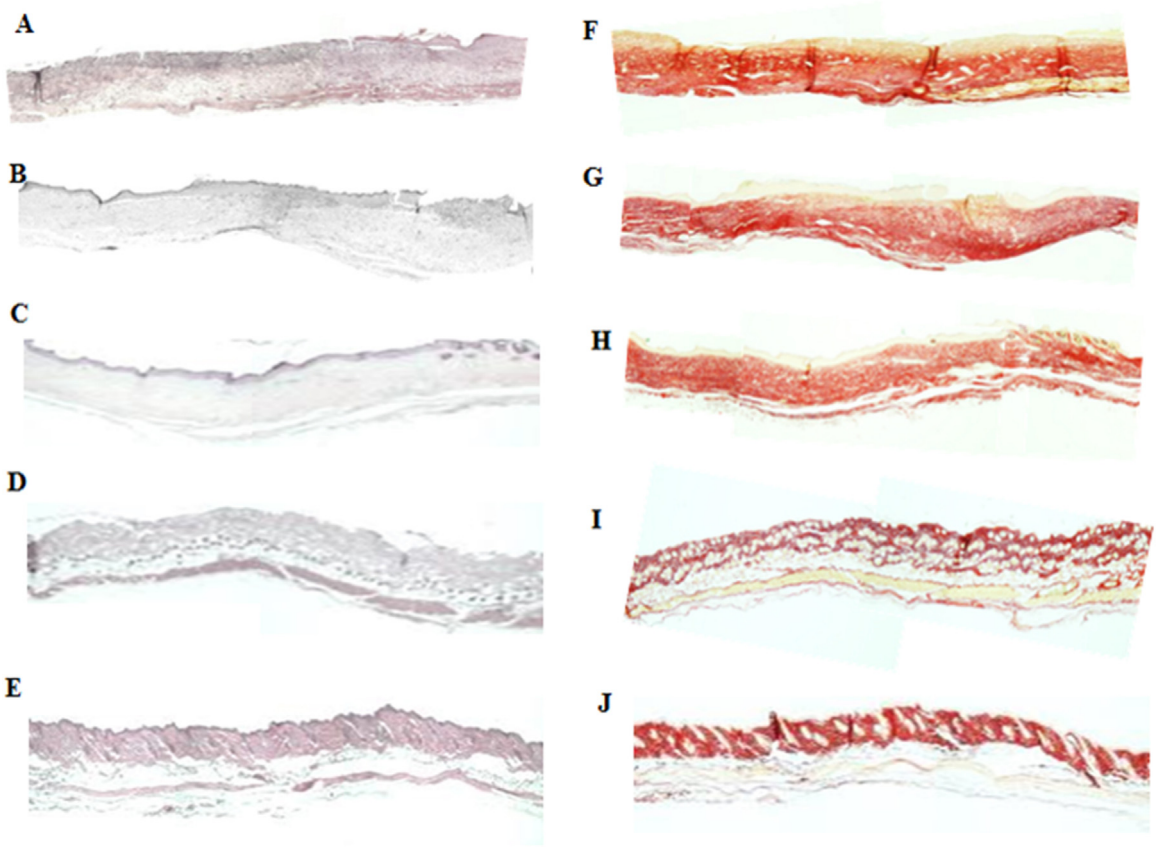

Fig. 5. Representative histological images of skin wounds by hematoxylin and eosin (H&E) staining (A–E) and Sirius Red staining (F–J) at day 21 post wounding. A, F: non-treated; B, G: hEGF solution; C, H: hEGF loaded PEG hydrogel; D, I: hEGF loaded heparin-based hydrogel; E, J: Normal mouse skin. Scale bale = 1 mm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.12. Immunochemistry staining

Sectioned tissues were also stained with an anti-wide spectrum cytokine antibody (rabbit polyclonal, 1:75, Abcam, Cambridge, MA, USA) by ABC staining using LSAB + system-HRP kit (DAKO, carpinteria, CA, USA) to observe the formation of keratinocyte layer at the wound site. The stained tissues were then counter-stained with Mayer's hematoxylin (Sigma, St.Loius, MO, USA). Macrophage staining was carried out by using an anti-macrophage antibody (mouse monoclonal, 1 μg/mL, Abcam, Cambridge, MA, USA).

2.13. Statistical analysis

Statistical analysis was conducted using analysis of variance (ANOVA) test. $P < 0.05$ was set to be a statistically significant value.

3. Results and discussion

3.1. Preparation and characterization of hydrogel sheets

A schematic diagram of preparing heparin-based hydrogel sheet is presented in Fig. 1(A). In this study, heparin-based hydrogel sheet was fabricated by thiol-ene reaction between thiolated heparin (Hep-SH) and diacrylated PEG (PEG-DA) upon UV irradiation. Heparin-based hydrogel is transparent in appearance as shown in Fig. 1(B).

The gel contents of photo-crosslinked hydrogel sheets after extraction were evaluated (Table 1). Both heparin-based hydrogel sheet and PEG hydrogel sheet remained over 95% of their original weight, suggesting that both types of hydrogel sheets were almost completely cross-linked. To further confirm the chemical crosslinking occurred in the heparin-based hydrogel, FT-IR analysis was carried out (Fig. S1). According to the PEG-DA spectrum, the absorption of C=C double bonds occurs at 1633 cm^{-1} and that of carbonyl groups occurs at 1724 cm^{-1} (Mohammad Imani, 2007). FTIR spectrum of Hep-SH presented a new peak at 2650 cm^{-1} compared to free heparin, which is attributed to the stretching vibration of S-H bonds. This indicates that cysteamine was successfully conjugated to the free heparin (Shi et al., 2013). The peak of double bonds of PEG-DA at 1633 cm^{-1} and that of S-H bonds of Hep-SH at 2650 cm^{-1} disappeared in the heparin-based hydrogel spectrum, indicating the consumption of the mentioned bonds during the photocrosslinking reaction (Bae, Gemeinhart, Divan, Suthar, & Mancini, 2010). Thus, FTIR analysis confirms that heparin-based hydrogel was successfully formed by photo polymerization *via* thiol-ene reaction.

The swelling capacity of hydrogels is closely related to crosslinking density. There was no noticeable difference between heparin-based hydrogel sheet and PEG hydrogel sheet in term of the swelling ratio due to their similar degree of chemical crosslinking (Table 1). Besides, it has been reported that PEG hydrogel and heparin-based hydrogel at the same concentration have the relatively similar network structure (Choi, Kim, Tae, & Kim, 2008b). Fig. 2. shows the swelling behaviors of heparin-based and PEG hydrogel sheets, and both hydrogel sheets were swelled very quickly in PBS within 5 min, but PEG gel sheets were equilibrated slowly compared to heparin gel sheet, probably due to the crystallization of PEG hydrogel during drying. It is crucial for wound dressings to absorb exudate quickly because it can provide the quick hemostatic ability and prevent the accumulation of exudates at the wound bed. The ability of heparin-based hydrogel sheet to

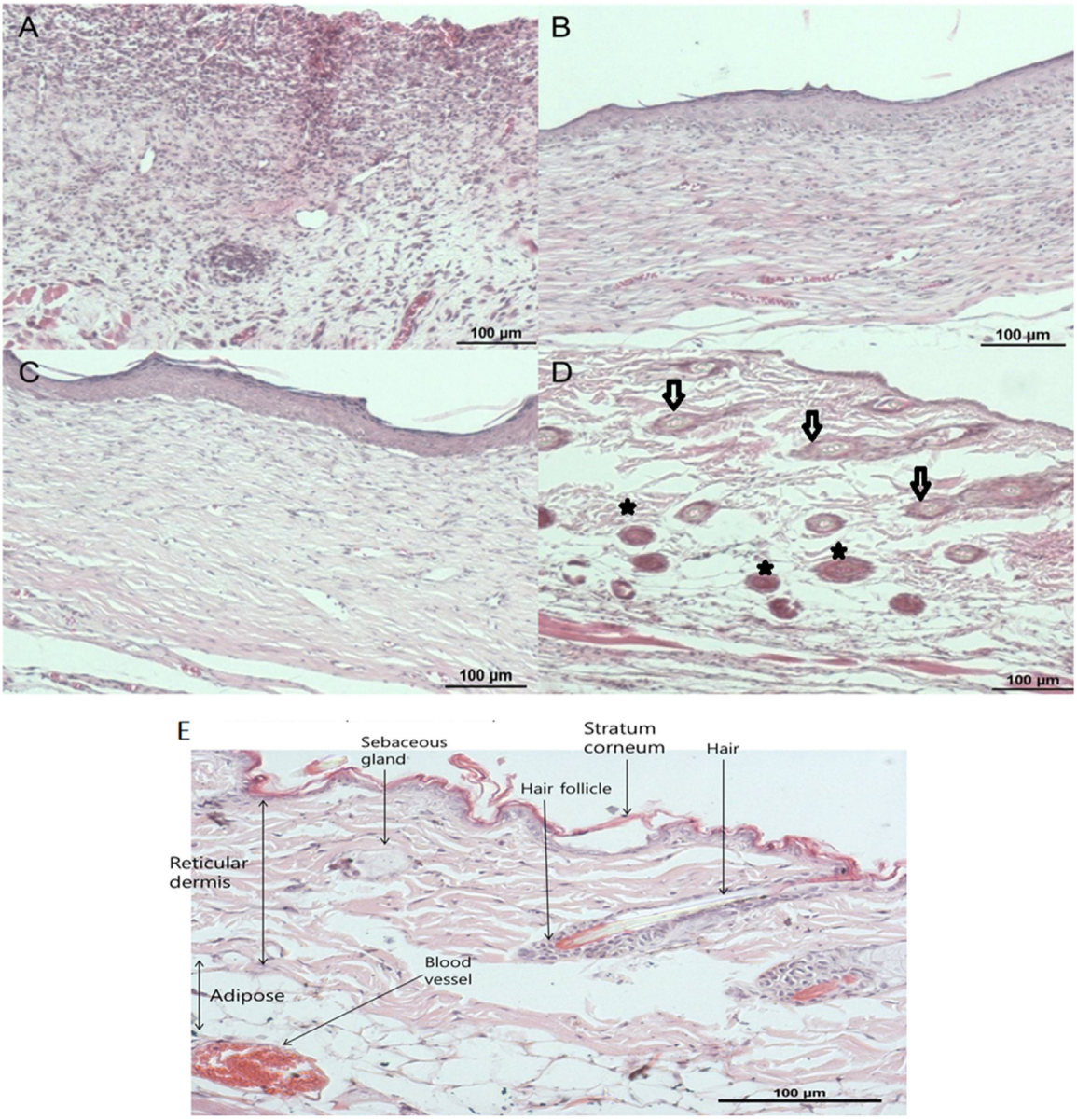

Fig. 6. Magnified images of H&E staining of mouse skin at day 21 post wounding. A: non-treated; B: hEGF solution; C: hEGF loaded PEG hydrogel; D: hEGF loaded heparin-based hydrogel; E: Normal mouse skin; Star: Blood vessels; Arrow: Hair follicle. Scale bar = 100 μm.

absorb body exudates quickly makes it suitable to be used as a wound dressing.

Hydrogels have been studied widely as a wound dressing due to their capability of absorbing a large amount of biological fluid without dissolution, thus giving them physical properties similar to soft tissues (Lin et al., 2015). Storage modulus (G') of hydrogel sheets before and after swelling was measured using a Rheometer. There were no significant differences between heparin-based hydrogel sheet and PEG hydrogel sheet in term of storage modulus (G') in either cases of before or after swelling (Table 1). Since the gel content of both types of hydrogel sheet was similar (Table 1), they were expected to have a similar storage modulus. The storage modulus of both types of hydrogel sheet before swelling was ~14 kPa which was similar to those of the skin in various positions in humans, indicating that they exhibited favorable mechanical properties as a wound dressing (Lu, Yu, Huang, Huang, & Zheng, 2009) (Pailler-Mattei, Bec, & Zahouani, 2008).

It has been reported that the evaporative water loss for normal skin, first degree burn, and granulating wound were 204 ± 12, 279 ± 26, and $5138 \pm 202\ g/m^2/day$, respectively. An idea wound dressing should allow adequate water vapor transmission with WVTR in the range of 2000–2500 $g/m^2/day$, because it can prevent both excessive dehydration as well as buildup of exudates at the wound bed (Queen, Gaylor, Evans, Courtney, & Reid, 1987). Commercially available hydrogel wound dressings have been shown to cover a wide range of WVTR, ranging from 120 (Duoderm CGF) to 9360 $g/m^2/day$ (Geliperm 1)(Wu et al., 1995). An excessive WVTR could lead to the total wound dehydration and scar formation, whereas a low WVTR will cause the accumulation of exudates, which might retard the healing process and increase the risk of bacterial growth (Kokabi, Sirousazar, & Hassan, 2007). The WVTRs of heparin-based hydrogel sheet and PEG hydrogel sheet were 1010 ± 100 and $1080 \pm 50\ g/m^2/day$, respectively, which are in the satisfactory range for this purpose and comparable to the commercial products.

Fig. 7. Immunohistochemical staining for cytokeratin of treated mouse skin at day 21 post wounding. Keratin is shown in brown and other tissues were counter-stained with hematoxylin. A: non-treated; B: hEGF solution; C: hEGF loaded PEG hydrogel; D: hEGF loaded heparin-based hydrogel; E: Normal mouse skin. Scale bar = 1 mm.

3.2. In vitro *human epidermal growth factor (hEGF) release from hydrogel sheets*

To maximize the effect of growth factors in damaged skin tissue regeneration, many delivery system have been studies for efficiently delivering wound caring growth factors to the wound site, including coacervate (Johnson & Wang, 2013), hydrogels (Choi & Yoo, 2010), and electrospun nanofiber (Choi, Leong, & Yoo, 2008).

Fig. 3 shows the release profile of hEGF from heparin-based hydrogel and PEG hydrogel. An initial burst of release was found for both types of hydrogel sheets. A complete release in 5 days was observed from PEG hydrogel sheet, whereas less than 50% of loaded hEGF was released in 5 days from heparin-based hydrogel sheet, followed by a very slow release over 21 days. Since both PEG and heparin-based hydrogel sheets have similar swelling ratios and modulus, similar release profiles would be expected if hEGF release were dominated by diffusion only (Choi et al., 2008b). However, heparin-based hydrogel sheet could hold and display hEGF more efficiently than PEG hydrogel sheet, suggesting the interaction between heparin-based hydrogel sheet and loaded hEGF. The overall release profile of hEGF from heparin gel was similar to that from other heparin gel formed by a visible light-initiated crosslinking with eosin Y (Fu et al., 2015), also supporting the major role of heparin in the release behavior. A relatively high concentration of heparin molecules in the heparin-based hydrogel sheet seems to contribute to the effective holding of hEGF in the heparin-based hydrogel in spite of relatively weak interaction of individual heparin molecule with hEGF compared to that with other growth factors.

3.3. In vivo *wound healing*

Wound contraction usually occurs between the first and second week after injury. Fast wound closure can prevent infection, dry state, and further tissue trauma at the injury site (Singer & Clark, 1999). The therapeutic efficacy of the hydrogel sheets was assessed by observing the wound closure as a function of time. Fig. 4A shows the preparation scheme of hEGF loaded hydrogel and transplantation of the hydrogel sheet to animal for the *in vivo* wound healing experiment.

Significantly accelerated closure of wounds treated with hEGF loaded heparin-based hydrogel sheet was evident by day 7 and continued throughout the test period compared to all other groups (Fig. 4B and C). After two weeks, the wounds treated with hEGF loaded heparin-based hydrogel sheet achieved significant closure to ~90%, compared to all other groups, which showed ~50–60% wound closure. The hEGF loaded in heparin-based hydrogel sheet would be less vulnerable to the attack by proteases due to the effective holding inside the hydrogel, and be released as a bioactive state, thus could continuously stimulate the proliferation and differentiation of keratinocytes and other cells. The hEGF loaded in PEG hydrogel sheet would be completely released within 5 days according to our *in vitro* hEGF release study (Fig. 3). The released hEGF would be then easily degraded by numerous abundant proteolytic enzymes in the wound sites and would become inactivated (Chen & Mooney, 2003). Topical application of hEGF would face the same degradation and inactivation problems as hEGF loaded in PEG hydrogel sheet, hence there was no significant difference in the wound closure rate for both cases on day 7. Since hEGF is mainly required during the initial state of wound healing process (Choi et al., 2008a,b), the non-treated control group showed the similar wound closure rate to the treatment group with only hEGF at day 7 and throughout the test period. Covering wound sites with hydrogel sheets yielded better wound closure rate compared to the non-treated group and only hEGF solution treated group. This could be attributed to the moisture environment at the wound site provided by hydrogel sheets, therefore accelerating the proliferation of keratinocytes and fibroblasts (Winter, 1962). For this reason, hydrogel systems had been studied widely as wound dressing to provide moisture-rich environments to epidermal cells.

3.4. *Histological examination*

Re-epithelization of covering the wound surface with new skin, neovascularization in which vascular integrity is restored to the region, and granulation repairing the structure integrity of the tissue defect by filling it with new connective tissue are the main and diverse processes that take place in the proliferative phase (Strodtbeck, 2001). To compare the degree of wound healing, H&E staining of mouse skin tissue was conducted in 21 days post wounding for all treatment groups by comparing to normal mouse skin tissue. In Figs. 5 and 6 , the control non- treated group did not produce completely closed epidermal layer and the formation of many granulocyte at the central area was observed due to the unclosed epidermal layer. Closure of wound surface is necessary to create a hypoxia wound environment and let the neovascularization to proceed (Clark, 1988). The hEGF solution only and hEFG loaded PEG hydrogel groups showed a closed epidermal layer and regenerated extracellular matrix. However, there was no blood vessel, hair follicle, or sebaceous gland formation. In contrast, the skin tissue treated with hEGF loaded heparin-based hydrogel sheet has a denser epithelialization, blood vessel, hair follicle, and sebaceous gland formation, as shown in Figs. 5D and 6D. In addition, the structure of the mouse skin tissue treated by hEGF loaded heparin-based hydrogel sheet was similar to that of the normal mouse skin tissue (Fig. 6E). This histological observation demonstrates that the wound healing of the hEGF loaded heparin-based hydrogel sheet was better than in the other groups and was very successful.

During the wound healing process, collagen is continuously synthesized for weeks or months after injury, and collagen and ECM tissue continue to be deposited into the wound (Strodtbeck, 2001). Collagen synthesis and deposition were visualized by Sirius Red staining (Fig. 5F–J). The non-treated control, hEGF solution only, and hEGF loaded PEG hydrogel groups (Fig. 5F–H) showed much thicker staining of collagen layer than hEGF loaded heparin-based hydrogel group (Fig. 5I). When the repaired connective tissue is not well anchored to the underlying connective tissue matrix, it is usually thicker than the normal skin (Strodtbeck, 2001). Thus,

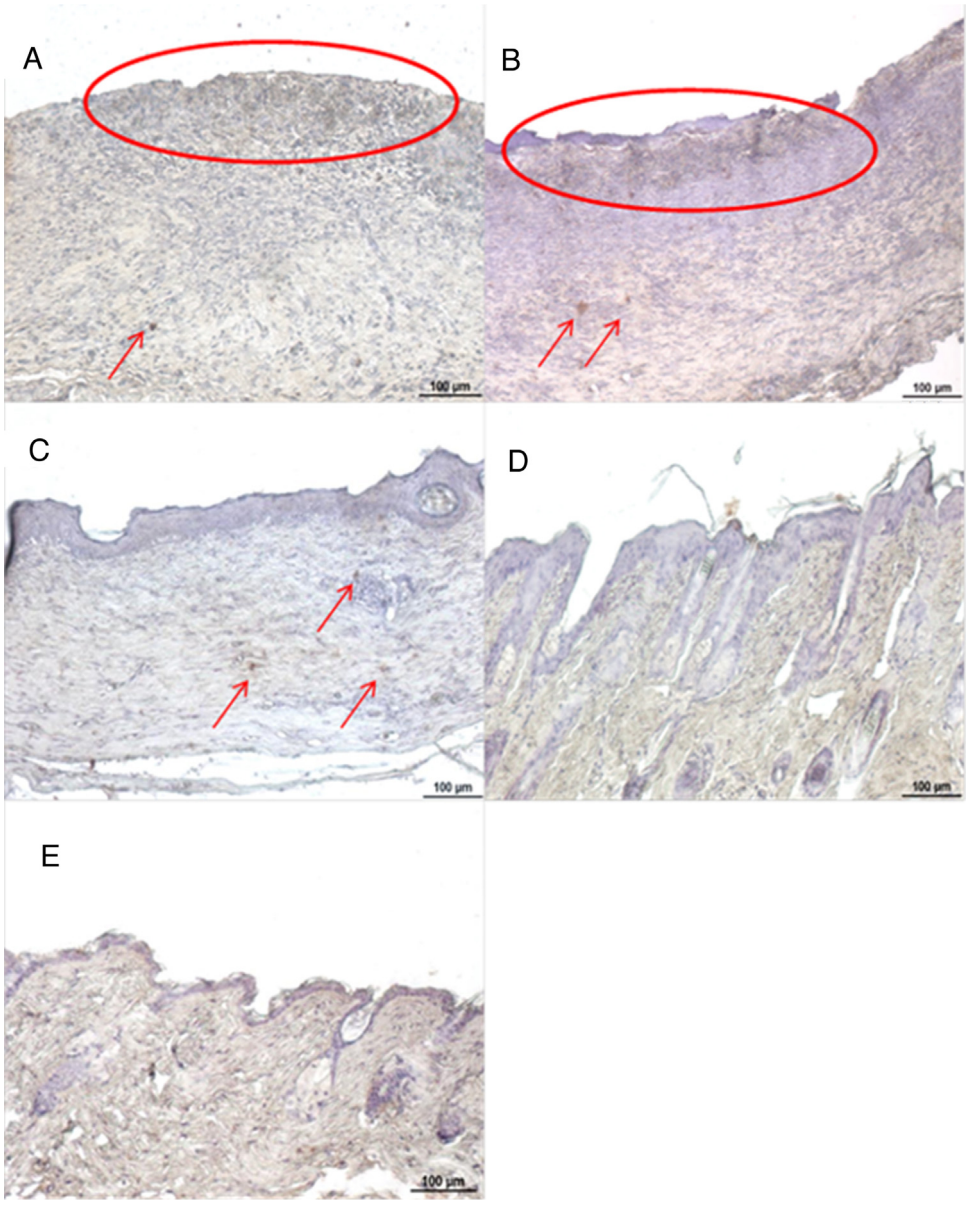

Fig. 8. Immunohistochemical staining for macrophage of treated mouse skin at day 21 post wounding. Other tissues were counter-stained with hematoxylin. A: Non-treated; B: hEGF solution; C: hEGF loaded PEG hydrogel; D: hEGF loaded heparin-based hydrogel; E: Normal mouse skin. Scale bar = 100 μm.

the thicker collagen layers stained in non-treated, hEGF solution only, and hEGF loaded PEG hydrogel groups seemed to represent the highly disorganized collagen structure throughout the granulation tissue where the progress to convert into a stable ECM was still going on (Velnar, Bailey, & Smrkolj, 2009). In contrast, structure of stained collagen layer of hEGF loaded heparin-based hydrogel group was similar to that of the normal skin (Fig. 5I and J), suggesting that the healing is at the final stage of remodeling phase where new collagen matrix becomes more oriented and cross-linked over time. Besides, the content of collagen of the treated mouse skins was determined by hydroxyproline assay as hydroxyproline is a characteristic amino acid in collagen (Lai et al., 2011). The hydroxyproline content of the wound skin treated by hEGF loaded heparin-based hydrogel was found to be similar to that of the normal skin sample and significantly higher than those of the other groups. (Fig. S2.). Also, there were no significant differences in the level of hydroxyproline for the wound skin treated by the other groups. Therefore, hEGF loaded heparin-based hydrogel sheet not only accelerated the wound closure rate but also facilitated the remodeling of the wound to normal skin tissue.

3.5. Immunohistochemical examination

Keratinocytes are the major epithelial cell in epidermis. Keratinocytes play a crucial roles in the re-epithelialization of a wound. A successful epithelialization of a wound occurs when keratinocytes completely cover the surface of the skin defect (Strodtbeck, 2001). It has been established that EGF enhances kerationocytes migration, thus promotes re-epithelialization and shortens healing time (Chen et al., 1993). To assess the effect of hEGF on keratinocytes migration and proliferation during the wound healing process, treated mouse skin tissues at day 21 post wounding were immunologically stained with an anti-wide spectrum cytokeratin antibody. In Fig. 7A–D, dark brown layer at epidermis was observed in all groups. Among them, the recovered tissue treated with hEGF loaded heparin hydrogel sheet (Fig. 7D) was intensely stained brown and even showed the hair follicles

formation, suggesting that keratinocytes proliferation and differentiation was predominant, presumably by the sustained release of hEGF from the heparin hydrogel sheet. In addition, hydrogels provided the moist environment and hence improved the migration of keratinocytes to the injury site because migration of the keratinocytes requires a fluid environment. The faster the wound closure, the faster remodeling could be induced (Strodtbeck, 2001). As shown in Fig. 4B, the hEGF loaded heparin hydrogel sheet accelerated the wound closure significantly from day 7 compared to others groups. This result supported the denser brown staining and even hair follicles formation at the recovered tissue.

Macrophages also play a crucial role in the wound healing process. Recruited macrophages during the diverse phases of skin repair exert specific functions to restore the tissue integrity (Lucas et al., 2010). Macrophages appear to be important in the early stage and mid stage of healing process as they enhance the wound closure rate, formation of vascularized granulation tissue, and epithelialization. However, at the last stage of the wound healing process, macrophages will undergo apoptosis and hence the amount is reduced (Rodero & Khosrotehrani, 2010). At day 21 post wounding, macrophages were detected on the surface of wound skin tissue in the non-treated control and hEGF solution only group (Fig. 8A–B). This indicates that proliferation stage of the wound still in the progress as macrophages are needed in producing cytokines or growth factors associated with re-epithelialization. In contrast, a very small amount of macrophages were detected in the central part of the wound treated by hEGF loaded PEG, and more importantly, there was no macrophages detected in wound treated by hEGF loaded heparin-based hydrogel as shown in Fig. 8C–D. This observation was similar to the normal skin where only 1–2 macrophages per 1 mm^2 are found (Delavary, van der Veer, van Egmond, Niessen, & Beelen, 2011). The minimal number of macrophages observed for hydrogel groups also indicates the inflammation and immune response of the hydrogels, supporting the proper biocompatibility of the hydrogel required for wound dressing (Lamb, Modjtahedi, Plant, & Ferns, 2004).

4. Conclusion

Biocompatible heparin-based hydrogel sheet prepared by thiolene and photo cross-linking reaction of thiolated heparin and diacrylated poly (ethylene glycol) provided an effective holding of loaded human epidermal growth factor. Heparin-based hydrogel sheet has a quick water uptake ability, suitable mechanical strength to serve as a wound dressing, and the water vapor transmission rate comparable with commercial product. hEGF loaded heparin-based hydrogel sheet significantly improved the wound healing effect compared to the non-treated control group, hEGF solution only, and hEGF loaded PEG hydrogel sheet. This study suggests the potential of hEGF loaded heparin-based hydrogel sheet for effective wound healing.

Acknowledgements

This research was financially supported by the “GIST-Caltech Research Collaboration Project” through a grant provided by GIST, Korea, the “GRI(GIST Research Institute)” Project through a grant provided by GIST in 2016, and by Ministry of Culture, Sports and Tourism (MCST) and Korea Creative Content Agency (KOCCA) in the Culture Technology (CT) Research & Development Program 2015.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2016.03.072.

References

Ajji, Z., Mirjalili, G., Alkhatab, A., & Dada, H. (2008). Use of electron beam for the production of hydrogel dressings. *Radiation Physics and Chemistry*, *77*(2), 200–202.
Ando, Y., & Jensen, P. J. (1993). Epidermal growth factor and insulin-like growth factor I enhance keratinocyte migration. *The Journal of Investigative Dermatology*, *100*(5), 633–639.
Bae, M., Gemeinhart, R. A., Divan, R., Suthar, K. J., & Mancini, D. C. (2010). Fabrication of poly(ethylene glycol) hydrogel structures for pharmaceutical applications using electron beam and optical lithography. *Journal of Vacuum Science & Technology B: Microelectronics and Nanometer Structures*, *28*(6). C6P24–C6P29
Bennett, N. T., & Schultz, G. S. (1993). Growth factors and wound healing: biochemical properties of growth factors and their receptors. *The American Journal of Surgery*, *165*(6), 728–737.
Chen, J. D., Kim, J. P., Zhang, K., Sarret, Y., Wynn, K. C., Kramer, R. H., et al. (1993). Epidermal growth factor (EGF) promotes human keratinocyte locomotion on collagen by increasing the alpha 2 integrin subunit. *Experimental Cell Research*, *209*(2), 216–223.
Chen, R. R., & Mooney, D. J. (2003). Polymeric growth factor delivery strategies for tissue engineering. *Pharmaceutical Research*, *20*(8), 1103–1112.
Choi, J. S., Leong, K. W., & Yoo, H. S. (2008). In vivo wound healing of diabetic ulcers using electrospun nanofibers immobilized with human epidermal growth factor (EGF). *Biomaterials*, *29*(5), 587–596.
Choi, J. S., & Yoo, H. S. (2010). Pluronic/chitosan hydrogels containing epidermal growth factor with wound-adhesive and photo-crosslinkable properties. *Journal of Biomedical Materials Research. Part A*, *95*(2), 564–573.
Choi, W. I., Kim, M., Tae, G., & Kim, Y. H. (2008a). Sustained release of human growth hormone from heparin-based hydrogel. *Biomacromolecules*, *9*(6), 1698–1704.
Choi, W. I., Kim, M., Tae, G., & Kim, Y. H. (2008b). Sustained release of human growth hormone from heparin-based hydrogel. *Biomacromolecules*, *9*(6), 1698–1704.
Delavary, B. M., van der Veer, W. M., van Egmond, M., Niessen, F. B., & Beelen, R. H. J. (2011). Macrophages in skin injury and repair. *Immunobiology*, *216*(7), 753–762.
Fu, A., Gwon, K., Kim, M., Tae, G., & Kornfield, J. A. (2015). Visible-light-initiated thiol–tcrylate photopolymerization of heparin-based hydrogels. *Biomacromolecules*, *16*(2), 497–506.
Gailit, J., & Clark, R. A. (1994). Wound repair in the context of extracellular matrix. *Current Opinion in Cell Biology*, *6*(5), 717–725.
Gainza, G., Bonafonte, D. C., Moreno, B., Aguirre, J. J., Gutierrez, F. B., Villullas, S., et al. (2015). The topical administration of rhEGF-loaded nanostructured lipid carriers (rhEGF-NLC) improves healing in a porcine full-thickness excisional wound model. *Journal of Controlled Release*, *197*, 41–47.
Hori, K., Sotozono, C., Hamuro, J., Yamasaki, K., Kimura, Y., Ozeki, M., et al. (2007). Controlled-release of epidermal growth factor from cationized gelatin hydrogel enhances corneal epithelial wound healing. *Journal of Controlled Release*, *118*(2), 169–176.
Johnson, N. R., & Wang, Y. (2013). Controlled delivery of heparin-binding EGF-like growth factor yields fast and comprehensive wound healing. *Journal of Controlled Release*, *166*(2), 124–129.
Kim, M., Kim, S. E., Kang, S. S., Kim, Y. H., & Tae, G. (2011). The use of de-differentiated chondrocytes delivered by a heparin-based hydrogel to regenerate cartilage in partial-thickness defects. *Biomaterials*, *32*(31), 7883–7896.
Kim, M., Kim, Y. H., & Tae, G. (2013). Human mesenchymal stem cell culture on heparin-based hydrogels and the modulation of interactions by gel elasticity and heparin amount. *Acta Biomaterialia*, *9*(8), 7833–7844.
Kim, M., Lee, J. Y., Jones, C. N., Revzin, A., & Tae, G. (2010). Heparin-based hydrogel as a matrix for encapsulation and cultivation of primary hepatocytes. *Biomaterials*, *31*(13), 3596–3603.
Kim, M., Shin, Y., Hong, B.-H., Kim, Y.-J., Chun, J.-S., Tae, G., et al. (2010). In vitro chondrocyte culture in a heparin-based hydrogel for cartilage regeneration. *Tissue Engineering. Part C, Methods*, *16*(1), 1–10.
Kokabi, M., Sirousazar, M., & Hassan, Z. M. (2007). PVA–clay nanocomposite hydrogels for wound dressing. *European Polymer Journal*, *43*(3), 773–781.
Laato, M., Niinikoski, J., Lebel, L., & Gerdin, B. (1986). Stimulation of wound healing by epidermal growth factor. A dose-dependent effect. *Annals of Surgery*, *203*(4), 379–381.
Lai, H. Y., Lim, Y. Y., & Kim, K. H. (2011). Potential dermal wound healing agent in Blechnum orientale Linn. *BMC Complementary and Alternative Medicine*, *11*, 62.
Lamb, D. J., Modjtahedi, H., Plant, N. J., & Ferns, G. A. A. (2004). EGF mediates monocyte chemotaxis and macrophage proliferation and EGF receptor is expressed in atherosclerotic plaques. *Atherosclerosis*, *176*(1), 21–26.

Lin, Y.-J., Lee, G.-H., Chou, C.-W., Chen, Y.-P., Wu, T.-H., & Lin, H.-R. (2015). Stimulation of wound healing by PU/hydrogel composites containing fibroblast growth factor-2. *Journal of Materials Chemistry B*, *3*(9), 1931–1941.

Lu, M.-H., Yu, W., Huang, Q.-H., Huang, Y.-P., & Zheng, Y.-P. (2009). A hand-held indentation system for the assessment of mechanical properties of soft tissues in vivo. *IEEE Transactions on Instrumentation and Measurement*, *58*(9), 3079–3085.

Lucas, T., Waisman, A., Ranjan, R., Roes, J., Krieg, T., Müller, W., et al. (2010). Differential roles of macrophages in diverse phases of skin repair. *The Journal of Immunology*, *184*(7), 3964–3977.

Mohammad Imani, S. S. (2007). Monitoring of polyethylene glycol-diacrylate-based hydrogel formation by real time NMR spectroscopy. *Iranian Polymer Journal*, *16*(1), 13–20.

Pailler-Mattei, C., Bec, S., & Zahouani, H. (2008). In vivo measurements of the elastic mechanical properties of human skin by indentation tests. *Medical Engineering & Physics*, *30*(5), 599–606.

Peles, Z., & Zilberman, M. (2012). Novel soy protein wound dressings with controlled antibiotic release: mechanical and physical properties. *Acta Biomaterialia*, *8*(1), 209–217.

Queen, D., Gaylor, J. D., Evans, J. H., Courtney, J. M., & Reid, W. H. (1987). The preclinical evaluation of the water vapour transmission rate through burn wound dressings. *Biomaterials*, *8*(5), 367–371.

Rodero, M. P., & Khosrotehrani, K. (2010). Skin wound healing modulation by macrophages. *International Journal of Clinical and Experimental Pathology*, *3*(7), 643–653.

Schultz, G. S., & Wysocki, A. (2009). Interactions between extracellular matrix and growth factors in wound healing. *Wound Repair and Regeneration*, *17*(2), 153–162.

Shah, S. S., Kim, M., Cahill-Thompson, K., Tae, G., & Revzin, A. (2011). Micropatterning of bioactive heparin-based hydrogels. *Soft Matter*, *7*(7), 3133–3140.

Shi, Y., Zhang, H., Yue, Z., Zhang, Z., Teng, K.-S., Li, M.-J., et al. (2013). Coupling gold nanoparticles to silica nanoparticles through disulfide bonds for glutathione detection. *Nanotechnology*, *24*(37), 375501.

Singer, A. J., & Clark, R. A. (1999). Cutaneous wound healing. *The New England Journal of Medicine*, *341*(10), 738–746.

Strodtbeck, F. (2001). Physiology of wound healing. *Newborn and Infant Nursing Reviews*, *1*(1), 43–52.

Sudheesh Kumar, P. T., Lakshmanan, V.-K., Anilkumar, T. V., Ramya, C., Reshmi, P., Unnikrishnan, A. G., et al. (2012). Flexible and microporous chitosan hydrogel/nano ZnO composite bandages for wound dressing: in vitro and in vivo evaluation. *ACS Applied Materials & Interfaces*, *4*(5), 2618–2629.

Tae, G., Kim, Y.-J., Choi, W.-I., Kim, M., Stayton, P. S., & Hoffman, A. S. (2007). Formation of a novel heparin-based hydrogel in the presence of heparin-binding biomolecules. *Biomacromolecules*, *8*(6), 1979–1986.

Thomas, S., & Hay, P. (1995). Fluid handling properties of hydrogel dressings. *Ostomy/Wound Management*, *41*(3), 54–56, 58–59.

Velnar, T., Bailey, T., & Smrkolj, V. (2009). The wound healing process: an overview of the cellular and molecular mechanisms. *The Journal of International Medical Research*, *37*(5), 1528–1542.

Vernon, T. (2000). Intrasite gel and intrasite conformable: the hydrogel range. *British Journal of Nursing (Mark Allen Publishing)*, *9*(16), 1083–1088.

Winter, G. D. (1962). Formation of the scab and the rate of epithelization of superficial wounds in the skin of the young domestic pig. *Nature*, *193*, 293–294.

Wu, P., Fisher, A. C., Foo, P. P., Queen, D., & Gaylor, J. D. (1995). In vitro assessment of water vapour transmission of synthetic wound dressings. *Biomaterials*, *16*(3), 171–175.

Yang, C., Xu, L., Zhou, Y., Zhang, X., Huang, X., Wang, M., et al. (2010). A green fabrication approach of gelatin/CM-chitosan hybrid hydrogel for wound healing. *Carbohydrate Polymers*, *82*(4), 1297–1305.

Yang, J. M., Yang, S. J., Lin, H. T., Wu, T.-H., & Chen, H.-J. (2008). Chitosan containing PU/Poly(NIPAAm) thermosensitive membrane for wound dressing. *Materials Science and Engineering: C*, *28*(1), 150–156.

Yang, X., Liu, Q., Chen, X., Yu, F., & Zhu, Z. (2008). Investigation of PVA/ws-chitosan hydrogels prepared by combined γ-irradiation and freeze-thawing. *Carbohydrate Polymers*, *73*(3), 401–408.

Clark, R. A. F. (Ed.). (1988). *The molecular and cellular biology of wound repair.*. Boston, MA: Springer US. http://dx.doi.org/10.1007/978-1-4899-0185-9. Retrieved from