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Hydrogen generating patch improves skin cell viability, migration activity, and collagen expression

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ABSTRACT

Molecular hydrogen recently attracted lots of attention in biomedical community because of its abilities to modulate signal transduction, protein phosphorylation, and gene expression that enable its anti-inflammatory, anti-allergy, and anti-apoptotic activities. However, controllable and local delivery of hydrogen molecules to tissues still remains challenging. Here, we propose a new method of delivering molecular hydrogen directly to human skin cells by designing a hydrogen-generating patch that releases hydrogen in surrounding environment as a product of chemical reaction of aluminum and carbon hydroxide in presence of water. We show that in the presence of molecular hydrogen the cells viability, expression of collagen, and migration efficiency of cells increase, thus suggesting that hydrogen can be used to promote better and faster healing of skin issues such as wounds and bruises.

Introduction

Molecular hydrogen has been shown to act as an important physiological regulatory factor with antioxidative, anti-inflammatory, and anti-apoptotic effects on cells composing various biological tissues and organs [1-5]. Consequently, many efforts were recently directed to designing and testing the hydrogen-based therapy. Notably, in the majority of previous studies, the hydrogen was usually delivered as a food or water supplement. As a result, this minimized the effectiveness of hydrogen delivery directly to the area of the treatment needs thus emphasizing the necessity for a new approach for hydrogen generation.

Positive effect of hydrogen on wound healing process has been demonstrated in animal models and human pilot studies [3-7]. However, in these studies, hydrogen was delivered to the skin either via inhalation, intravenous injection of hydrogen infused saline, or consumption of hydrogen-rich water. These methods of delivery of hydrogen could be used only in special medical facility and by medical personal and / or offer very limited control of an administered dose.

In previous studies, prominent positive effects of the hydrogen-rich water treatment were documented in experiments conducted with a human skin [4,5] and animal skin [6,7] though no success in localizing the hydrogen delivery was demonstrated before. We addressed this challenge and reported a new method of local H_2 delivery to the human skin cells by designing a hydrogen-generating patch that can be attached directly to the skin [8]. The hydrogen generation was activated by adding water to the mixture of aluminum and calcium hydroxide powders that promotes chemical release of hydrogen through the plastic packaging of the patch. Our results demonstrated increase in hydrogen concentration in the surrounding cell culture medium up to 350 ppb that remains stable over 6 hours duration. Presence of molecular hydrogen in the cell culture media improved human skin cells viability, increased production of collagen, and enhanced the cells migration ability. Our results demonstrate a new concept for implementing the hydrogen therapy and provide a promising route for hydrogen use in treating skin damages.

Experimental procedure

Preparation of Hydrogen generating powder

Hydrogen generation powder as a major component of the patch was prepared to stabilize the effectiveness and duration of hydrogen release to surrounding environment. For this, purchased from Sigma Aldrich aluminum (~5 μm particle size) and calcium hydroxide ($Ca(OH)_2$, ~3 μm particle size) powders were mixed by ball-milling in 1:6 relative

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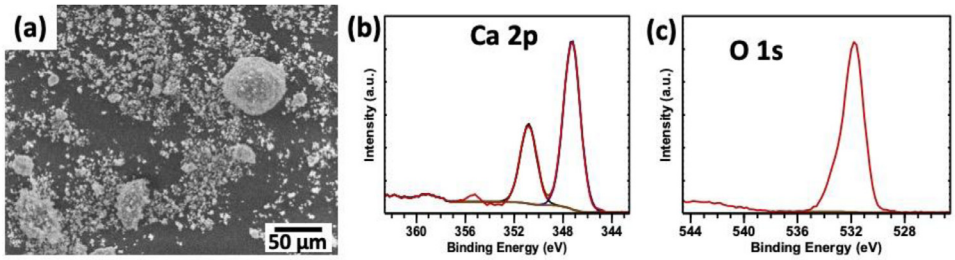

Fig. 1. (a) Scanning electron microscopy (SEM) image and x-ray photoemission spectroscopy analysis of (b) Ca and (c) O1s spectra.

weight concentration to create a non-active hydrogen-generating powder (Fig. 1). Calcium hydroxide powder has a tendency to agglomerate thus slowing down the hydrogen release and prolonging the operation life of the patch once water is added to the powder and reaction with aluminum and water is initiated. Hydrogen generation was activated immediately prior to the tests by adding double distilled (DD) water to the dry powder mixture. A detailed overview of the hydrogen release mechanism is provided below.

Upon water adding, a water suspension of food-grade calcium hydroxide, $Ca(OH)_2$, "slaked lime", is created. When the suspension is mixed with aluminum powder, the following set of chemical reactions is activated:

$$Al_2O_3 + 3Ca(OH)_2 = Ca_3\left(AlO_3\right)_2 + 3H_2O,$$

$$2Al + 6H_2O = 2Al(OH)_3 + 3H_2 \uparrow,$$

$$2Al + 3Ca(OH)_2 = Ca_3\left(AlO_3\right)_2 + 3H_2 \uparrow,$$

and

$$2Al(OH)_3 + Ca(OH)_2 = Ca\left[Al(OH)_4\right]_2.$$

Note that metallic Al is usually covered by a thin layer of aluminum oxide. The first reaction describes the removal of a thin film of aluminum oxide present of the surface of aluminum powder. The second and the third reactions lead to the release of molecular hydrogen and this release proceeds until all metallic aluminum is consumed. For a complete third reaction, the ratio of the weight of aluminum to calcium hydroxide is 1:4, However, in the patch, we used a ratio 1:6 due to the low solubility of calcium hydroxide in water. Reactions occur with a small amount of heat, which is insignificant for the experiment.

Preparation of Hydrogen generating patch

To control the release of hydrogen and avoid exposure of cells to other components of the reactive mixture, the hydrogen generating patch (Patch Model 19-1) was designed. Specifically, the procedure was as following: a) 150 mg of powder was added into the low-density polyethylene (LDPE) bag (patch) with a cotton mesh inside, b) 3 ml of DD water was added directly to the patch to activate the set of chemical reactions c) the patch was sealed using an impulse bag sealer (Medtronic) to prevent leaking of the mixture to the surrounding environment; d) the patch was immersed in the cell culture media for further measurements. PE material for the patch design was chosen based on previous reports indicating high hydrogen diffusion through the PE film [9].

In addition to hydrogen-generating patches, similar patches but only with calcium hydroxide powder (without aluminum powder) were designed for control tests (Control patch). During the control tests, similar procedure of water addition and sealing of the patch was used.

Preparation of Hydrogen-Rich Media

Serum-free EMEM media was treated with hydrogen by submerging Hydrogen-generating patch into the 50 mL centrifuge tube with 25 mL media and incubating this media with the patch for 6 hours. For the control experiments a Control patch was submerged in the tube with the media. To treat the cells with Hydrogen 0.1 mL of the treated media was aliquoted and placed into each well of 96-well plate. For the Control 0.1 mL of Control Hydrogen-untreated media was used.

Cell viability test

BJ fibroblast cells (CRL2522, ATCC) after incubation were seeded onto 96-well plate at 5000 cells per well. Cells were cultured overnight at 37°C and 5% CO_2. After the incubation the original medium was discarded and 0.1 mL of serum-free EMEM media treated with Hydrogen patch or Control patch was added into each well, 6 wells for each treatment. Serum-free Hydrogen-treated or Control EMEM media was replenished every 2 h to maintain the same hydrogen level which was measured and confirmed using a Trustlex ENH-2000 Hydrogen Meter (http//www.trustlex.co.jp). Potential heat generation and temperature increase were negligible as confirmed by the temperature measurements. The described above experiment design allowed avoiding any direct contact of the cells with the Hydrogen or Control patches and therefore eliminated a potential heat effect on the cells. The media was replenished in each well three times and, after 6 hours, the media was discarded from each well and cell viability test was performed with MTT reagent according to the protocol described earlier [10].

Model of wound closure

For the wound closure experiment, BJ fibroblast cells (CRL2522, ATCC) were cultured in the 6-well plate in Eagle's Minimum Essentia Medium (EMEM, ATCC) for 24 hours to reach 90% confluence. Wound was created by 1000 μL tips. Pictures of wound were taken using an objective with 4× magnification at the beginning of the experiment. Then, the medium was changed to the serum-free EMEM treated by H_2-generation patch or Control patch. These media were prepared in 50 mL centrifuge tube by placing the H_2-generating patch or the Control patch inside 25 mL of media as presented in Fig. 1(a). Medium extract treated with H_2-generating patch or Control patch was added to the 6 well plates after taking pictures at different time point. The width of wound was measured by ImageJ software.

The rate of wound closure was calculated as following:

Rate of wound closure = (initial width - current width) / time period.

Collagen expression with Western Blot

After wound closure experiment, cells from 6-well plate were harvested for protein extraction and Western Blot analysis. After measuring protein concentrations, protein samples were loaded into SDS-PAGE gel, and then transferred on to the polyvinylidene difluoride (PVDF) membrane following instructions. The PVDF membrane was blocked with 5% milk for 2 h at room temperature and incubated overnight with rabbit polyclonal anti-Collagen I primary antibody (Abcam) at 4°C. Then the secondary antibody, Donkey anti-rabbit IgG (H+L) from Jackson ImmunResearch Laboratories Inc., was added to the primary-antibody treated PVDF membrane and incubated for 2 hours at room temperature.

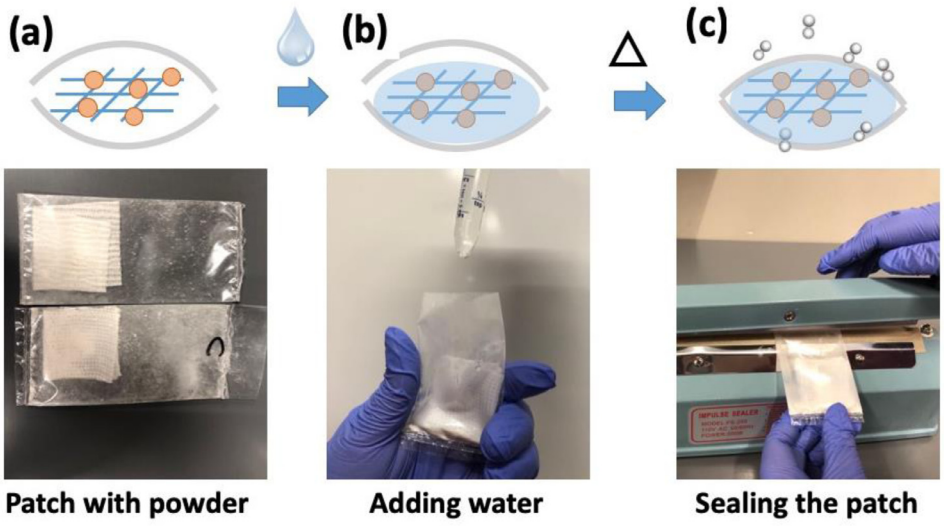

Fig. 2. Hydrogen patch preparation process. (a) Cotton mesh with the powder is placed in the plastic patch. (b) Water added to the patch directly before the experiment. (c) The patch filled with mixture of the powder and water is sealed using the vacuum sealer. Letter "c" indicates the controlled patch without addition of aluminum powder.

Pictures were taken using Bio-Rad imaging system. Band intensity was measured with ImageJ software and analyzed with GraphPad software. β-actin was used as an expression control.

Power of the Study and Statistical Analysis

The one-way analysis of variance (ANOVA) and two independent-samples t-tests using SPSS Statistics were used to analyze the data. In both types of tests, samples after exposure to hydrogen-rich and hydrogen-deficient culture media were analyzed with $P<0.05$ to indicate levels of statistical significance.

Results and discussion

The steps of the hydrogen-generating (or control) patch preparation are summarized in Fig. 2: (a) the LDPE patches were loaded with the hydrogen-generating (or control) powders, (b) water was added to the hydrogen-generating patch to activate the hydrogen generation or to the control patch for the control test, (c) the air was removed from the patch as much as possible and the patch was sealed to isolate the powder mixture from the environment.

The stability of the hydrogen release in the cell culture media treated with the hydrogen patch was tested by was tested by Trustlex ENH-2000 Hydrogen Meter. The continuous stable generation of hydrogen was observed for about 6 hours (Fig. 3). The hydrogen concentration started to decline after 6 hours indicating reduced rate of chemical reaction producing molecular hydrogen. The release of the hydrogen and its concentration during the tests were monitored using Trustlex ENH-2000 Hydrogen Meter. Stability of the hydrogen concentration is attributed to two competing processes, hydrogen generation and hydrogen dissociation from culture media to surrounding ambient environment [11]. Once the hydrogen generation rate reduces due to complete conversion of aluminum powder into stable compounds with calcium, the hydrogen dissociation to atmosphere dominates thus reducing hydrogen concentration in cell culture media. The results of this hydrogen concentration monitoring experiment were used to define the operational lifetime of the patch model (Hydrogen patch) and establish the experimental parameters for testing the wound closure effectiveness and the cell viability. During the experiments with the cell culture the release of the hydrogen and its concentration during were monitored in the culture media using Trustlex ENH-2000 Hydrogen Meter.

After establishing the effectiveness and duration of the hydrogen release, we focused on exploring the bio effectiveness of hydrogen in improving the viability of skin cells. For this, after the incubation, the skin

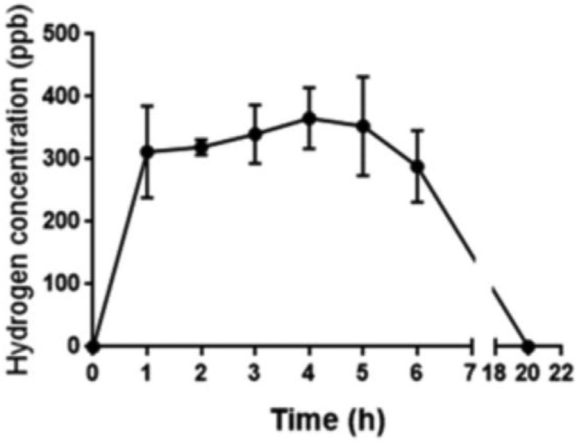

Fig. 3. Measurements of hydrogen generation efficiency in cell culture media treated with Hydrogen-generating patch. The figure summarizes the hydrogen concentration measured in the cell culture medium as a function of time starting immediately after adding water to the powder and sealing the patch. The results indicate stable hydrogen concentration of ~350 ppb over a 6 hour period.

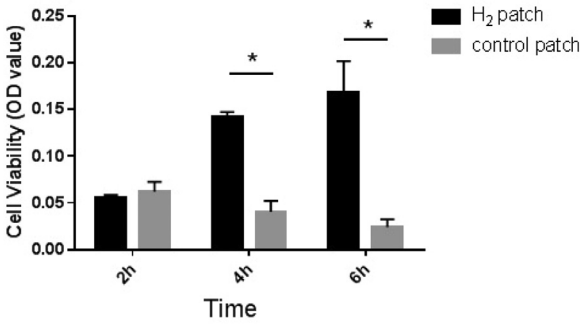

Fig. 4. Cell viability hydrogen-treated cells and control cells when exposed to the serum-free media. The results indicate increased OD number for hydrogen-treated cells in contrast to control test.

cells were transferred to two wells of the serum-free media, one well was with the hydrogen-rich media (hydrogen-patch) and another well was with hydrogen-deficient media (control test). The cell viability results obtained using the MTT assay are summarized in Fig. 4. The cells exposed to the media with control patch (hydrogen-deficient media)

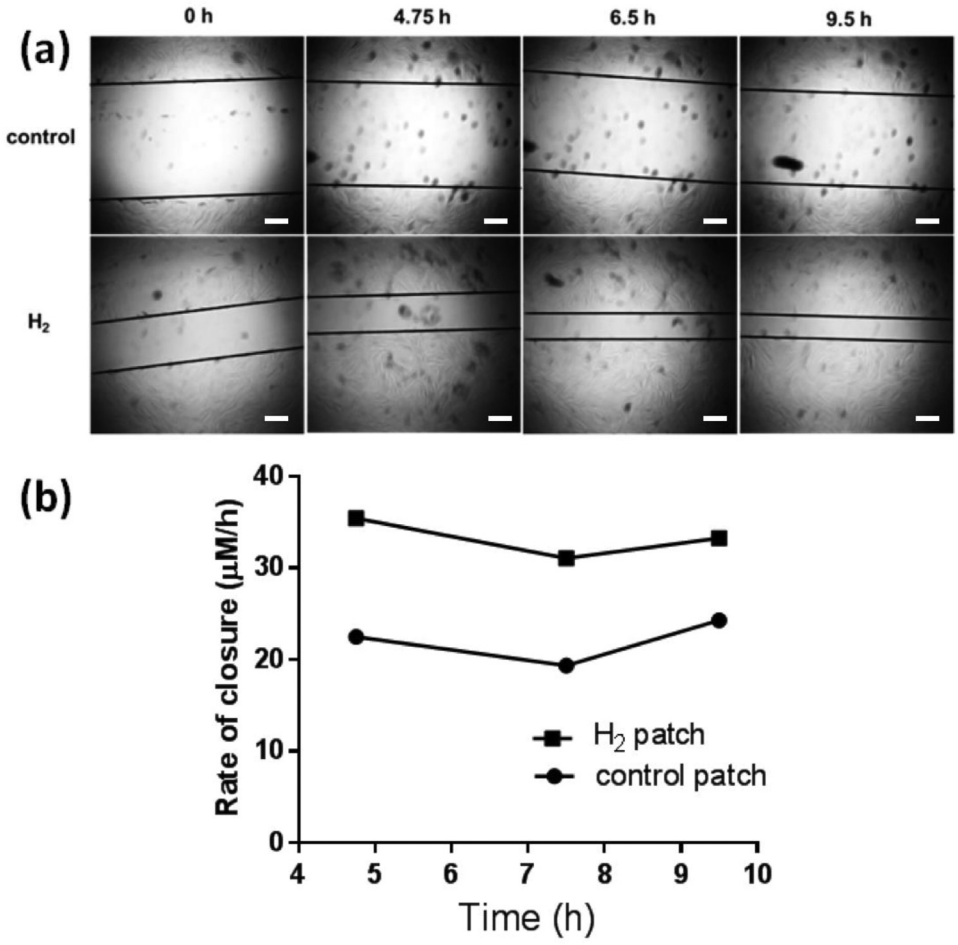

Fig. 5. Wound closure assay of hydrogen-treated cell and control cells. (a) Optical images of the wounds closures in case of control and hydrogen-treated cells as a function of time. (b) Comparison of wound closure rated for hydrogen-treated cells and control cells. The scale bar is 250 μm.

demonstrate reduction in optical density (OD) number as it is expected for serum-free media [12]. Meanwhile, treatment of cells with hydrogen increases OD number even in the serum-free media. These results confirm the beneficial effect of hydrogen on the skin cells viability.

To test the potential applicability of hydrogen patch to promote curing of skin damage such as a wound, we also tested the migration activity of the cells. For this, we replicated the wound by scratching the cells of the well-plate. The cells migration to close the wounds in hydrogen-rich and hydrogen-deficient environments was monitored over 9.5 hours (Fig. 5). From optical images (Fig. 5a) one can see that cells treated with hydrogen had higher migration resulting in a more efficient wound closure. The changes in the width of the wound (Fig. 5b) indicate that cell treated with hydrogen had about two times higher rate of closure as compared to control test when the cells were exposed to the hydrogen-deficient media. These results suggest that hydrogen do not only promotes the viability of the cells but also accelerates the cell migration to heal the damage.

The growth of tissue around the wound site involves not only the angiogenesis process, when endothelial cells migrate in the area of the wound, but also the collagen production or deposition by these cells [13,14]. The acceleration of the collagen deposition is important because it increases the strength of the wound and allows cells involved in inflammation, angiogenesis, and connective tissue construction to grow and differentiate on the collagen matrix laid down by fibroblasts [15]. Initially Type III Collagen and fibronectin are produced, but later Collagen III is replaced by the stronger Collagen I [13].To complement the wound closure experiment, we also tested the level of the collagen expression in hydrogen-treated and control cells. Fig. 6 summarizes western blot analysis results. The results indicate that collagen expression

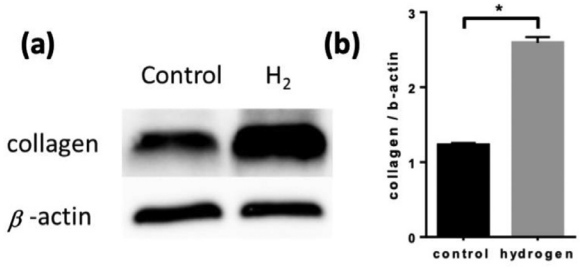

Fig. 6. Collagen expression in hydrogen-treated cells and control cells. (a) Collagen expression level in hydrogen-treated cells and control cells ($P<0.05$). (b) Calculated relative collagen/β-actin values for the hydrogen-treated cells and control calls.

level is significantly higher in cells treated with hydrogen as compared to control cells. This suggests promoted skin cell repair activity in presence of hydrogen thus further confirming the effectiveness of the proposed hydrogen patch in treating the skin damage.

It has been demonstrated that upon *in-vitro* aging the keratocyte migration into collagen gel matrix significantly declines which may impair corneal wound healing [16]. Increased viability of the cells is therefore expected to further improve the wound healing process. These results indicate the hydrogen potential for effective and simple wound healing as an alternative approach to previously reported nanocomposite cryogels [17].

Conclusion

The repair of wounds is a complex biological process that involves activation and synchronization of multiple biological pathways. Here, we proposed a new method for local and effective delivery of hydrogen to promote bioactivity of the human skin cells and to accelerate the wound healing process. For this, we created a hydrogen generating patch that can be directly applied to the skin to enable localization of the hydrogen delivery to the skin cells. Design of the patch based on the chemical reaction initiated in the mixture of aluminum, calcium hydroxide, and water, allowed controlled and prolonged release of hydrogen over the 6 hour duration. We demonstrated that hydrogen release from the patch promoted cell viability, cell migration, and collagen release. These results indicate the hydrogen potential for better wound healing thus indicating that hydrogen patch can be used for effective treatment of skin damage.

Declaration of Competing Interests

No competing interests were disclosed.

CRediT authorship contribution statement

Marina Safonov: Conceptualization, Methodology, Data curation, Writing - original draft. **Jing You:** Data curation, Writing - original draft. **Jihyung Lee:** Data curation. **Vladimir L. Safonov:** Investigation, Writing - original draft. **Diana Berman:** Supervision, Conceptualization, Writing - original draft. **Donghui Zhu:** Supervision, Validation, Resources.

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References

[1] I. Ohsawa, M. Ishikawa, K. Takahashi, Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals, et al Nature Medicine 13 (2007) 688–694.
[2] S. Ohta, Recent progress toward hydrogen medicine: potential of molecular hydrogen for preventive and therapeutic applications, Current Pharmaceutical Design 17 (2011) 2241–2252.
[3] G.L. Nicolson, G.F. de Mattos, R. Settineri, Clinical effects of hydrogen administration: from animal and human diseases to exercise medicine. Int. J, et al Clinical Medicine 7 (2016) 32–76.
[4] S. Kato, Y. Saitoh, K. Iwai, N. Miwa, Hydrogen-rich electrolyzed warm water represses wrinkle formation against UVA ray together with type-I collagen production and oxidative-stress diminishment in fibroblasts and cell-injury prevention in keratinocytes, J Photochemistry and Photobiology B: Biology 106 (2012) 24–33.
[5] Q. Li, S. Kato, D. Matsuoka, et al., Hydrogen water intake via tube-feeding for patients with pressure ulcer and its reconstructive effects on normal human skin cells in vitro, Medical Gas Research 3 (2013) 20.
[6] W. Fang, Hydrogen gas inhalation protects against cutaneous ischaemia/reperfusion injury in a mouse model of pressure ulcer, J Cell Mol Med 22 (2018) 4243–4252.
[7] P. Zhou et.al, The healing effect of hydrogen-rich water on acute radiation-induced skin injury in rats, Journal of Radiation Research 60 (1) (2019) 17–22.
[8] V.L. Safonov, Systems and methods for topical application of molecular hydrogen, US Patent Application (2019) 0308001.
[9] M.H. Klopffer, B. Flaconneche, P. Odru, Transport properties of gas mixtures through polyethylene, Plastics, Rubber and Composites 36 (2007) 184–189.
[10] A. Shirani, Combined tribological and bactericidal effect of nanodiamonds as a potential lubricant for artificial joints, ACS Applied Materials and Interfaces 11 (2019) 43500–43508.
[11] X. Zhao, H. Jin, Investigation of hydrogen diffusion in supercritical water: A molecular dynamics simulation study, International Journal of Heat and Mass Transfer 133 (2019) 718–728.
[12] W. Strober, Trypan blue exclusion test of cell viability, (Current Protocols in Immunology) (2015) 111.
[13] G. Gurtner, S. Werner, Y. Barrandon, M.T. Longaker, Wound repair and regeneration, Nature 453 (2008) 314–321.
[14] V. Falanga, Wound Healing. American Academy of Dermatology (AAD), 2005.
[15] Z. Ruszczak, Effect of collagen matrices on dermal wound healing, Advanced Drug Delivery Reviews 55 (2003) 1595–1611.
[16] S.R. Sandeman, M.C. Allen, C. Liu, R.G. Faragher, A.W. Lloyd, Human keratocyte migration into collagen gels declines with in vitro ageing, Mechanisms of Ageing and Development 119 (2000) 149–157.
[17] X. Zhao, Guo B, H. Wu, Y. Liang, P.X. Ma, Injectable antibacterial conductive nanocomposite cryogels with rapid shape recovery for noncompressible hemorrhage and wound healing, Nature Communications 92784 (2018) 1–17.