Controlling pH by electronic ion pumps to fight fibrosis

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Fibrosis and scar formation is a medical condition observed under various circumstances, ranging from skin wound healing to cardiac deterioration after myocardial infarction. Among other complex interdependent phases during wound healing, fibrosis is associated with an increased fibroblast to myofibroblast transition. A common hypothesis is that decreasing the pH of non-healing, alkaline wounds to a pH range of 6.0 to 6.5 increases healing rates. A new material-based strategy to change the pH by use of electronic ion pumps is here proposed. In contrast to passive acidic wound dressings limited by non-controlled delivery kinetics, the unique electronic ion pump design and operation enables a continuous regulation of pH by H\textsuperscript{+} delivery over prolonged durations. In an in vitro model, fibroblast to myofibroblast differentiation is attenuated by lowering the physiological pH to an acidic regime of 6.62 ± 0.06. Compared to differentiated myofibroblasts in media at pH 7.4, gene and protein expression of fibrosis relevant markers α-smooth muscle actin and collagen 1 is significantly reduced. In conclusion, myofibroblast differentiation can be steered by controlling the pH of the cellular microenvironment by use of the electronic ion pump technology as new bioelectronic drug delivery devices. This technology opens up new therapeutic avenues to induce scar-free wound healing.

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1. Introduction

The wound healing process is characterised by highly complex and interdependent phases, involving haemostasis, inflammation, migration, proliferation, and maturation. While the majority of acute wounds heal without complications within 8 to 12 weeks, non-healing, chronic, or in general “complex wounds” are a considerable challenge with respect to treatment and cure [1,2]. In addition to primary effects such as low healing rates or secondary effects such as bacterial infection, skin wound fibrosis and pronounced scar formation present a high prevalence, affecting over 100 million patients each year [3]. These effects not only cause significant physical and psychological distress to individual patients, in particular after occurrence of burn wound contractures [4], but also impose a high financial burden to the healthcare system. Thus, there is a clear demand for an increased understanding of the wound healing process and its underlying mechanisms, as well as for new therapeutic strategies and technologies for a variety of wound healing scenarios. In particular, it is crucial to achieve a functional technology for wound healing, which expresses therapeutic factors in a dynamic and highly addressable fashion in order to optimise efficacy, and to minimise scar formation.

While the sustained presence of myofibroblasts with increased collagen type 1 deposition and excessive extracellular matrix remodelling have been identified as the main hallmark of fibrosis and scarring [5,6], no effective therapeutic approach is currently available to address the onset of fibrosis- despite tremendous research that has been conducted during the last decade [6,7].

In recent years, the importance of wound pH, its spatial changes across wounds or temporal fluctuations during wound closure have been appreciated [8–11]. Non-healing, chronic wounds are characterised by a pH value of up to 8.9, whereas an acidic pH value of 6.0 to 6.5 was shown in a healthy state during wound healing. It has been hypothesised that increased healing rates and wound closure of hard to heal wounds can be achieved by decreasing the pH level of wounds to a non-alkaline condition [9,10]. Underlying...
mechanisms are widely unknown, yet acidic pH values around 6.5 within the wound bed have been reported to act manifold: (i) neutralising toxic bacterial metabolites (which can be alkaline), (ii) inducing angiogenesis, (iii) affecting matrix metalloproteinase activity, and (iv) influencing keratinocyte proliferation and fibroblast activity and differentiation [9,10]. Thus, acidic, or in general, functional wound dressings are an interesting concept for the treatment of fibrotic wounds.

Material-controlled delivery of active substances has the potential to provide local dosage at the point of interest for therapeutic agents, which may have detrimental side-effects when administered systemically [12]. Thus, therapies for wound healing utilising drug-loaded dressings have been investigated extensively [13]. However, conventional drug-loaded material approaches often suffer from considerable burst release, inherent to the chosen matrix for delivery [14]. Specifically, diffusion-driven drug delivery processes are often characterised by the release of an initially high concentration of the active compound, followed by rapidly changing kinetics and lower concentration profiles [14]. Further, drug release from passive wound dressings cannot be controlled externally. Importantly, however, responsive and controlled drug release is necessary to most effectively meet the needs of the complex and changing local environments within a wound [15]. Stimuli responsive materials that provide on-demand release in response to changes in temperature, pH, or other factors have the potential to address some of these challenges, and introduce an important feedback into the dynamic wound healing process [15,16]. Nevertheless, detailed spatiotemporal control over active drug concentration profiles, including the wound pH, remains a challenge. To address the inherent drawbacks of conventional drug delivery approaches, a new device paradigm based on bioelectronic technologies is increasingly being explored [17–19]. Analogous to modern electronic chip design and fabrication, these bioelectronic devices employ material combinations and microscale patterning techniques of ionically conducting polyelectrolytes and conjugated polymers to realise sophisticated device structures that enable electronic control over the delivery of ionic compounds. Such bioelectronic devices are particularly promising in that bulky fluidic systems can be avoided and digitally controlled electronic signals can be used to steer delivery of bioactive compounds [17,20,21]. Further, with electronic addressing, health care solutions providing spatiotemporal release of active substances using a wide range of standard communication protocols, such as Bluetooth, NFC and WiFi, become also possible [22–24]. One such delivery technology are so-called (organic) electronic ion pumps, which have the capability of providing localised ion delivery at high dosage precision and rapid on/off switching in the complete absence of fluid flow [25,26]. A variety of electronic ion pump devices have been demonstrated to allow the delivery of a range of bioactive compounds, including the neurotransmitter acetylcholine to selectively stimulate nerve cells [27], γ-aminobutyric acid (GABA) to alleviate pain in an animal model [28,29], salicylic acid to control inflammatory responses of monocytes in vitro [30], and the plant hormone auxin to regulate plant physiological processes [31]. Despite these versatile demonstrations, electronic ion pump based technologies have typically been developed to interact with biological ionic signaling systems, and have not been previously explored as a strategy for the delivery of pharmaceutical compounds for wound treatment. Further, despite pH being recognised as a particularly important parameter in the wound healing process, highly specific and controlled H+ delivery to the site of interest has not been investigated as a therapy to date and we are not aware of any in vitro studies investigating the effect of environmental pH on fibrosis on the cellular level. Here, we suggest that controlling fibroblast to myofibroblast differentiation by actively maintaining a pH around 6.5 could be an avenue to address scar formation during wound healing. As such, an in vitro model system was designed to study the effect of electronic ion pump-regulated pH on the differentiation of human fibroblasts into myofibroblasts. Using the electronic ion pump as a platform technology, we demonstrate attenuated myofibroblast differentiation at an acidic pH. With this proof of concept study, we aim to highlight the importance of pH during this differentiation step and subsequently call attention to the unmet potential of regulating wound bed pH to fight scar formation.

2. Materials and methods

2.1. Chemicals and consumables

Cell culture plates were purchased from Techno Plastic Products AG (TPP, Switzerland). If not stated differently, cell culture supplements were purchased from Thermo Fisher Scientific, Switzerland. Cell culture media and all other chemicals were from Sigma, Switzerland, and used as received without any further purification.

2.2. Human dermal fibroblast culture

In vitro evaluation was carried out with normal human dermal fibroblasts (NHDF, female, Caucasian, skin/temple. C-29910, PromoCell, Germany) of passage 12 and lower. NHDF were expanded in Dulbecco’s Modified Eagle’s Medium (DMEM), high glucose, supplemented with 10% (v/v) fetal calf serum, 1% glutamine, and 1% penicillin, streptomycin, neomycin (PSN). Fibroblast to myofibroblast differentiation was induced in serum starved media (RPMI 1640), supplemented with 1% horse serum, 1% glutamine, 1% PSN, and 2 ng·mL⁻¹ transforming growth factor beta-1 (TGFβ1). An acidic medium was achieved by reduced buffer concentrations (sodium bicarbonate NaHCO₃) in RPMI 1640 media to maintain a pH of 6.5 in an environment with 5% CO₂. Acidic medium was supplemented with 1% horse serum, 1% glutamine, 1% PSN and 2 ng·mL⁻¹ TGFβ1. NHDF were cultured to sub-confluence in DMEM for 24 h after seeding, followed by incubation in the respective media for 8 h, 1 day, or 3 days. All experiments were performed under aseptic conditions and NHDF were cultured in a humidified incubator at 37°C with 5% CO₂. For electronic ion pump experiments, cells were cultured in NaHCO₃-free culture media in an incubator set to 0% CO₂ to avoid proton buffering upon delivery. To create an acidic environment during the evaluation phase, pH of the medium was adjusted by addition of 0.1 M HCl.

Cell metabolic activity, indicative for cell viability, was assessed with an alamarBlue assay (Life Technologies, Thermo Fisher Scientific, Switzerland). NHDF were incubated for 2 h at 37°C in 10% (v/v) alamarBlue in phenol red free medium. alamarBlue reduction was quantified based on fluorescent excitation and emission at λex = 580 nm, λem = 540 nm (Mithras² Plate reader, Berthold Technologies, Germany). DNA was quantified by a Hoechst assay, using a DNA-binding fluorescent agent (bis-benzimidazole, Hoechst 33258, Sigma). Cells were lysed in milliQ water by 3 thawing-freezing cycles. Cell lysates were then incubated with the Hoechst dye at room temperature for 1 h. Fluorescence was measured on a plate reader using an excitation wavelength at λex = 350 nm and emission at λem = 460 nm. All evaluations were accomplished in N=3 individual experiments with n=3 technical repeats.

2.3. Immunofluorescence analysis

NHDF were seeded at a density of 3000 cells per well in a 96-well plate and cultured as described above. NHDF were rinsed in phosphate buffered saline (PBS), fixed in 4% (v/v) formaldehyde in PBS for 30 min, followed by 3 washes in PBS. NHDF were then permeabilised for 15 min with 0.1% (v/v) TritonX™-100 in PBS, followed by blocking in bovine serum albumin (BSA 3% (w/v) in
PBS). Alpha smooth muscle actin (α-SMA) was labelled with a FITC-conjugated anti-α-SMA antibody (Sigma Aldrich, mouse monoclonal, clone 1A4, –2 µg·mL⁻¹ in PBS, overnight at 4°C). Actin was stained with phalloidin, Alexa 546®-conjugated (~0.03 µM in PBS, 1 h), nuclei were counterstained with 4’6-diamidino-2-phenylindole (DAPI, 1 µg·mL⁻¹ in PBS, 1 h). Images were acquired with a Confocal Laser Scanning Microscope (LSM780, Carl Zeiss AG, Germany). To stain extracellular collagen 1, NHDF were fixed in formaldehyde, washed in PBS and incubated with anti-collagen 1 antibody (Abcam, United Kingdom, –2 µg·mL⁻¹ in PBS overnight at 4°C), followed by incubation with an Alexa-555® conjugated anti-rabbit secondary antibody (~2.5 µg·mL⁻¹ in PBS, 45 min). NHDF were then permeabilised with TritonX-100 to stain for actin (phalloidin Alexa-633® conjugated), α-SMA, and nuclei, as described above.

2.4. Fluorescence activated flow cytometry

For quantitative analysis of α-SMA expression, NHDF were labelled with a FITC-conjugated anti-α-SMA antibody and the percentage of α-SMA-positive cells was quantified by flow cytometry. Briefly, NHDF were seeded at a density of 200,000 cells per flasks in T75 cultured cells flask as described above. NHDF were harvested and stained in suspension following the protocol described under 2.3. Each step was followed by centrifugation for 5 min at 250 x g and removal of the supernatant. Non-labelled cells as well as cells stained with an isotype control (IgG2a–FITC isotype control from murine myeloma) were used for calibration and background correction. Flow cytometry was accomplished on a Gallios Flow Cytometer (Beckman Coulter, Switzerland). For each condition, 10,000 cells were gated in forward/side scatter at a maximum of 15 min acquisition time. Data analysis and quantification was performed using the Kaluza software provided by the manufacturer. N=3 individual experiments.

2.5. Quantitative real time polymerase chain reaction (qRT-PCR)

Gene expression profiles were assessed by qRT-PCR on a CFX96 Thermal Cycler and Real-Time PCR Detection System (BioRad Laboratories Inc., USA). RNA isolation was accomplished with an RNeasy Micro Kit (Qiagen, Leica Microsystems GmbH, Germany) according to the manufacturer’s instructions. In brief, NHDF were cultured at a seeding density of 100,000 cells in T25 cell culture flasks under the respective conditions for 8 h, 1 day, or 3 days, trypsinised, centrifuged at 250 x g and lysed in RLT-buffer, supplemented with 1% (v/v) β-mercaptoethanol, prior to RNA extraction. RNA content was measured using a NanoDrop (ND-1000, Witec AG, Switzerland). For reverse transcription to cDNA, an iScript cDNA Synthesis Kit from BioRad was used. All primers (Table 1) were ordered from Microsynth AG, Switzerland. Gene expression was evaluated according to the 2-ΔΔCT method [32], with GAPDH as reference gene and normalisation to fibroblasts expanded in DMEM. N=3 individual experiments with n=3 replicates in each experiment.

2.6. Electronic ion pumps

Electronic ion pumps were manufactured utilising a previously reported PSS-co-MA/PEG polyelectrolyte synthesis [33], and a planar device structure on poly(ethylene terephthalate) (PET) substrates [31]. Briefly, 4-inch PET substrates were cleaned with acetone and water, followed by an oxygen plasma surface treatment. Immediately following, a solution of PSS-co-MA/PEG in H⁺ form (1:1, 4% (w/v) in deionized water to isopropanol) was mixed with 1.5% (w/v) poly(ethylene glycol) (PEG, Mw = ~400), and spin-coated at 1500 rpm. The thin film was baked at 110°C for 2 h. To increase the polyelectrolyte’s thickness (and thus lower the ion pump’s operating resistance), a second identical solution of PSS-co-MA/PEG was spin-coated and baked atop the first layer. Ion pump channels were patterned using Shipley S-1818 G2 photoresist and developed with Microposit MF319 following a standard photolithographic processing protocol. The exposed PSS-co-MA/PEG was removed via reactive ion etch (CF₄ and O₂, 150 W, 180 s), and the remaining patterned photoresist was stripped in acetone. An ion exchange in 200 mM HCl was then performed for 5 min. Ion pump channels were encapsulated by bar coating Dupont 5018 UV curable ink (2 × 25 µm), and devices were then cut-out by hand using scissors and scalpel. The individual free standing electronic ion pumps were fixed to an electrolyte reservoir (Hellermann Tyton shrink tubing, with adhesive lining, 35 mm), finally, the device reservoirs were filled with a solution of 0.1 M KCl and stored in a solution of the same until further use.

2.7. Electronic ion pump calibration and pH changes

Proton delivery is directly proportional to the applied current [26]. Theoretical values of pH changes at applied currents of 0.2, 0.3, or 0.5 µA were calculated based on Eqs. (1)–(3).

Definition of pH: \[ pH = -\log[H^+] \] (1)

Amount of delivered H⁺: \[ H^+ = e^- = \frac{I \times t}{F} \] (2)

Final pH: \[ pH_{final} = -\log[H^+]_{final} = -\log\left(\frac{H^+}{H^+_{delivered}}\right) = -\log\left(H^+_{initial} + \frac{I \times t}{F}\right) \] (3)

Where \( H^+ \) is the proton concentration in the target medium; \( F \) is Faraday's constant, 96485 C·mol⁻¹; \( I \) is the applied electrical current in A; and \( t \) is the time in s. OEIP function was tested by inserting electronic ion pumps into 150 µL of an electrolyte solution of 0.1 M KCl in a 48-well plate. pH was recorded at different time points after 0.5, 1, 2 or 3 h, respectively. 48-well plates were gently shaken before measuring the pH to enhance H⁺ distribution. Before in vitro experiments, the time of H⁺ delivery to reach a defined pH value was assessed in buffer-free medium in an incubator set to 0% CO₂. pH was measured at regular intervals until a value of ~6.5 was reached. pH was measured with a pH Meter 713 (Metrohm AG, Switzerland) equipped with a bioredox for small volume measurements. A three-point calibration of the device was performed prior to each experiment.

2.8. Controlled proton delivery to dermal fibroblasts

NHDF were cultured in 48-well plates in DMEM for 24 h prior to medium change (differentiation medium) and subsequent proton delivery (5000 cells per well). Plate lids were replaced by a
parafilm foil that was used to close the dish and an adhesive plate sealing foil on top of the parafilm for increased stability. Electronic ion pumps were inserted through small slots in this foil. The outlet at the tip of the pump was positioned in close proximity to the cell layer without touching the cells. A second electronic ion pump in the same well served as counter electrode. Reservoirs of proton delivering pumps were filled with sterile filtered 0.1 M HCl solution, while reservoirs of the counter electrodes were filled with sterile filtered 0.1 M KCl solution.

Electronic ion pumps were electrically addressed using Ag/AgCl electrodes (silver wire, 1 h in 1 M FeCl₃ solution) placed inside the reservoirs. The Ag/AgCl electrodes were then connected via electrical clamps to a Burster Digistant 6706 (Burster Präzisionsmesstechnik GmbH, Germany) that provided a constant current of 0.5 μA for 32 h (time point evaluated beforehand, see 2.6.2). The same setup, without applied current served as control (0.0 μA).

Devices were removed from the culture wells, and cells were maintained for an additional 3 days in the now acidic medium prior to immunofluorescent analysis as described above. The small cell number did not allow for quantification via flow cytometry. α-SMA expression was therefore quantified based on fluorescent images (from stitch tile images of 2.55 by 2.55 mm²). The cell area that was positively stained for α-SMA was quantified automatically with ImageJ (conversion to a binary image, followed by quantification of the white area without any additional threshold set). Values are normalised to the total cell number based on automatic nuclei counts. Results are presented as area (μm²) per cell, which gives a quantitative value to the microscopic immunofluorescence analysis. For each condition, at least n=9 images from N=3 individual experiments were analysed with a minimum of 500 nuclei counts per image.

2.9. Statistical analysis

Numerical values are reported as mean ± SD. Based on the relatively small sample size, normal distribution could not be assumed and non-parametric tests were used. Differences of the mean among more than two groups were assessed in a Kruskal Wallis test, followed by pair-wise comparison in a Mann-Whitney test. Differences among the groups with P values of < 0.05 were accepted as significant. Statistical analysis was performed with GraphPad.

3. Results and discussion

3.1. Electronic ion pumps allow for controlled proton delivery

Electronic ion pumps used in this study were designed in accordance with previous work and following earlier reports [31,33]. Briefly, the functional core unit of the ion pumps was based on PSS-co-MA/PEG, a polyelectrolyte ion exchange membrane (IEM). This class of polymer is characterised by a high fixed charge concentration, a material property that allows for charge selective ionic transport of atomic ions and small linear molecules at relatively high ionic conductance values. Further, the PSS-co-MA/PEG material system is compatible with common microfabrication processes, thus enabling a wide range of bioelectronic device architectures and addressable systems with micro scaled features.

Rather than a mechanical or (micro)fluidics-based approach, the drug delivery mechanism of electronic ion pumps involves the flow-free, electrophoretic transport of ions through one or more polyelectrolyte IEM channels. Put simply, the IEM channel consists of a polymer backbone that carries a high density of negative charges (in this case, making it a cation exchange membrane). By applying an electrical bias, charge transport is initiated through the IEM and H⁺ ions from the HCl reservoir will travel through the hydrated polymer to the other side where they are released into the culture medium. In contrast to conventional drug delivery methodologies, only the desired ion is delivered to the target site, restricting both the flow of other oppositely charged co-ions dissolved in the source electrolyte as well as the overall flow of solvent.

Organic electronic devices fabricated from these polyelectrolyte IEM materials – in the form of the electronic ion pump – can deliver a near arbitrary and precise chemical signal (delivery of an active compound) at the site of interest in direct proportion to the applied electronic signal. The electronic ion pump system developed and used in this study is illustrated in Fig. 1. A conventional shrink tubing formed the reservoirs for either HCl or KCl solutions, respectively. By using a second electronic ion pump to physically separate the counter electrode from the cell culture, we could guarantee the cytocompatibility of the system and did not impose additional effects arising from e.g. metallic counter electrodes or Ag/AgCl electrodes directly immersed in the cell culture well.

 Adequate functioning of each device and proton delivery (change in pH) in response to applied current was first evaluated in a standard electrolyte of 0.1 M KCl and compared to theoretical pH calculations based on Eq. (3) (Fig. 1C). The calculated delivery efficiency decreased with increasing current, with a 1.7 fold higher efficiency at 0.2 μA (58 ± 1 %) as compared to 0.5 μA (35 ± 0.7 %). These relatively low proton delivery efficiencies, as well as the observed efficiency in dependence on operating current, were unexpected. Generally, a low delivery efficiency can be attributed to a less-than~100% IEM selectivity, or device defects resulting in parasitic water channels. Such water channels result in a back flow of ions – from target to source – which contributes to a non-negligible parasitic ionic current to the total measured electronic current. While not explored further here, IEM stability and device efficiency remain active areas of investigation [34,35]. It has recently been reported that IEM selectivity can be greatly affected by IEM channel geometries and/or operational protocols (i.e. high ionic transport currents) [36,37]. Nevertheless, low delivery efficiencies were not considered to be a problem in these studies, as a direct correlation between applied current and pH (or delivery time and current) was observed here, and adequate proton delivery of each electronic ion pump used in subsequent cell culture could be guaranteed.

3.2. Cell culture is maintained at a pH of 6.5 without compromising cell viability

The hypothesis that an acidic environment has a beneficial effect on wound healing, and in particular reduces scar formation, has been investigated in an in vitro model with human dermal fibroblasts (NHDF) prior to the use of electronic ion pumps in cell culture. Experimental conditions were divided into three groups: (i) NHDF cultured in starvation media without the addition of TGFβ₁ (abbreviated as ctrl.), (ii) NHDF cultured in differentiation media with the addition of TGFβ₁ (abbreviated as diff.), or (iii) NHDF cultured under acidic conditions with a target pH value of 6.5 and the addition of TGFβ₁ (abbreviated as pH=6.5). Cell culture media formulations and CO₂ concentrations were adjusted in order to lower the pH to 6.5 and maintain an acidic milieu for extended culture periods. The sodium bicarbonate–carbon dioxide buffer-system is widely used in mammalian cell culture to maintain the pH at 7.4 [38]. CO₂ from the gaseous phase dissolves in the culture medium and is in equilibrium with sodium bicarbonate according to CO₂ + H₂O ⇌ HCO₃⁻ + H⁺. Taking advantage of this system, the pH can be shifted to lower values by reducing buffer concentrations at constant supply of CO₂. In an incubator with 5% CO₂, a concentration of 0.034 g·mL⁻¹ NaHCO₃ was sufficient to
reduce the pH to a value of 6.62 ± 0.06, while at standard buffer concentrations of 2.0 g•mL⁻¹, pH values were maintained at 7.37 ± 0.06 or 7.32 ± 0.03 in the ctrl. or diff. group, respectively (Fig. 2A). These established media formulations allowed for the culture of NHDF under neutral or acidic conditions without compromising the cell viability. Cell metabolic activity as well as DNA quantity exceeded 80% relative to the control group (ctrl.), which lies above the cytocompatibility threshold (70% of the control group) stated by the ISO-norm 10993-5. For experiments involving proton delivery by electronic ion pumps, NHDF were cultured in a humidified incubator that was set to a CO₂ concentration of 0% and in the absence of sodium bicarbonate, as the presence of standard buffer concentrations would quench proton delivery. Similar to the cell culture conditions with sodium bicarbonate in a 5% CO₂ environment, pH values were stable at 7.41 ± 0.03, 7.36 ± 0.08, or 6.62 ± 0.10 for the three respective media defined as diff., ctrl., or pH=6.5 (Fig. 2B). Cell metabolic activity as well as DNA content were above 80% relative to the standard condition presented in Fig. 2A. The effect of pH on viability of fibroblasts in vitro was previously investigated [39,40] and it was shown that fibroblasts tolerated a wide range of pH, ranging from mean values of 6.5 to 11.5, which is in agreement with our findings in the acidic regime and the media formulations defined in this study.

3.3. An acidic pH attenuates fibroblast to myofibroblast differentiation

pH has been recognised as an important factor in wound healing, affecting a variety of cellular and molecular mechanisms [11]. As with any drug or factor being studied in cell culture, it cannot be ruled out that the drug, in our case the H⁺ regulated change in pH, does affect a multitude of mechanisms or pathways. In the simplified in vitro model, here reported, we aimed at recapitulating key aspects of skin scarring, and decided to solely investigate one of the factors during fibrosis in an acidic environment, specifically, TGFβ1-induced differentiation of human dermal fibroblasts into myofibroblasts.

On the protein level, the expression of α-SMA and collagen type 1 was analysed based on immunofluorescence after three days in the respective medium (Fig. 3). The time point was chosen based on preceding experiments (data not shown) that confirmed TGFβ1-induced differentiation of fibroblasts into myofibro-
lasts after three days under these defined conditions. Longer culture times did not result in better discrimination between differentiated and non-differentiated fibroblasts. Differentiated myofibroblasts stained positive for α-SMA (green), with markedly increased expression compared to cells cultured under control conditions or at pH=6.5 (Fig. 3 A). α-SMA expression was furthermore quantified by flow cytometry, which confirmed the image-based observations (Fig. 3 B). In the group of differentiated myofibroblasts (diff.), significantly more cells stained positive for α-SMA (88.35 ± 4.78 %) compared to pH=6.5 (12.81 ± 12.77 %). In line with this expression, the second assessed hallmark for fibrosis, collagen type 1 deposition, was also enhanced in differentiated myofibroblasts. Under acidic conditions, however, collagen deposition was similar to non-differentiated fibroblasts. In line, it was observed that TGFβ1 supplemented media (diff.) significantly increased gene expression of α-SMA and collagen type 1 (coll1) compared to the control group (Fig. 3 C and E). Under acidic conditions, and continuous stimulation with TGFβ1, these values decreased compared to diff., showing a gene expression profile similar to non-differentiated fibroblasts in the control group (ctrl.). The expression of myofibroblast-specific genes was monitored over three days and while peak values were observed at the last time point, differences in expression levels were already apparent after one day, suggesting an early switch from the fibroblastic to myofibroblastic phenotype in this in vitro model.

Interestingly, Park et al. reported that both basic (pH>7.50) and acidic (pH<6.04) extracellular environments downregulated coll1 gene levels in human dermal fibroblasts in vitro [39]. Collagen deposition and extracellular matrix remodeling in general is a major player of wound healing, skin formation, and scar development [41] and it is therefore suggested that adjusting the pH can be an approach to control scarring by controlling collagen breakdown and deposition towards a more elastic and less fibrotic tissue. In addition to observations on collagen breakdown, the effect of acidic or basic pH in wound healing scenarios was previously investigated by Kruse and colleagues [40]. The authors reported on decelerated wound healing in vivo under prolonged acidic conditions, indicated by reduced fibroblast or keratinocyte migration, and epithelialisation compared to neutral conditions. Studies by Sharpe et al. point in the same direction, demonstrating that fibroblast and keratinocyte proliferation was most pronounced in an alkaline environment (pH of 7.2 to 8.3) [42]. Fibroblast to myofibroblast differentiation was, however, not investigated by the authors and it remains an open question, to what extent fibrotic pathways and scar formation are affected under acidic conditions. Interestingly, these experimental results are contrasting previous reviews where the benefits of acidic wound dressings have been discussed [9,10] and even further underpin the complexity of wound healing. The involved pathways and possible therapeutic strategies demand further research to elucidate the effect of pH on various mechanisms at the cellular level. Conflicting results are also found regarding skin pH in general, due to the lack of standardised measuring methods, and observed differences across gender and age [43].

3.4. Changing the pH by controlled H⁺ delivery to the cell culture

Based on this confirmed effect of attenuated fibroblast differentiation under acidic conditions (Fig. 3), proton delivery by electronic ion pumps and cell culture was combined. The ion pump outlets were superimposed with the cell layer grown on TCP. In a custom-made setup, four pairs of devices were connected in series, allowing for 4 replicates within one experiment. Power was sourced across all 8 electronic ion pump devices simultaneously, and the serial wiring ensured the same H⁺ ionic delivery current
to each well, irrespective of individual well or resistance values of the electronic ion pump. The setup is illustrated in Fig. 4.

In spite of previous investigations with electronic ion pumps in biomedical settings [27–31], their use in fibroblast cell culture has not been evaluated previously and proton delivery to this cell culture medium was a new challenge. In addition to technical considerations, cell culture media formulations were especially designed for this targeted proton delivery. The buffered system (sodium bicarbonate) of standard cell culture media has been adjusted to maintain a stable pH of 7.4 and is thus not appropriate to be used in evaluations where the pH should be changed by proton delivery via electronic ion pumps. As described under 3.2, a buffer-free, CO₂-free cell culture system that allowed maintaining a culture of viable NHDF was established. Subsequently, proton delivery and thus pH changes were assessed in buffer-free cell culture media in an incubator set to 0% CO₂. Repeated pH measurements at regular time intervals showed that at an applied current of 0.5 μA, a period of around 32 h was needed in order to reach a pH of 6.48 ± 0.11 (starting at an initial pH of 7.45) (Fig. 4 C). The pH linearly decreased with time (y = −0.03x + 7.46; R²=0.998), allowing to control the pH in the cell culture well in a volume of 150 μL by applied current and delivery time. Compared to a standard electrolyte (0.1 M KCl), in which the pH was lowered within a few hours (Fig. 1), cell culture media without sodium bicarbonate still had a strong buffering capacity, which can be attributed to the high concentrations of various nutrients, amino acids, and salts, necessitating a longer delivery time.

After proton supply by electronic ion pumps and having reached a pH value of 6.5, cells were maintained in culture for 3 more days, similar to the cell culture period defined above. Immunofluorescent analysis showed that at a current of 0.5 μA, only very few cells were positively stained for α-SMA. Cells cultured under differentiation conditions without electronic ion pumps (diff.) or ion pumps without applied current (0.0 μA), however, differentiated into α-SMA⁺ myofibroblasts (Fig. 5). Quantitative image-based analysis revealed significantly higher levels of α-SMA expression in these conditions compared to cells cultured under acidic conditions after proton delivery. The area that stained positive for α-SMA (green) was quantified based on image analysis and reported as μm² normalised to the cell number based on automatic nuclei count. Values are 304 ± 126 μm² (diff.), 336 ± 158 μm² (0.0 μA), or 55 ± 45 μm² (pH=6.5), respectively (Fig. 5D). These evaluations could confirm our findings of attenuated myofibroblast differentiation in an acidic environment displayed in Fig. 3, where the pH was lowered by a reduction in buffer in a 5% CO₂ environment and not by use of electronic ion pumps.

An externally controlled system with a rapid on/off switch (current supply) would also allow proton delivery in a defined pattern,
with varied concentrations at different intervals. This is particularly interesting to address the different phases of wound healing, during which fibroblast or keratinocyte proliferation or differentiation states could be addressed by changes in pH [42,43]. Feedback loop systems based on proton delivery devices coupled with adaptive machine learning were developed by Selberg and colleagues [44]. In a proof of concept study, a fluorescent reporter was used to adjust proton delivery or removal in response to the cell membrane potential. Such closed loop systems are tremendously promising for in vitro models of wound healing to address the different, highly complex phases. This level of precision could not feasibly be achieved by manually adjusting buffer concentrations or HCl addition in situ. Additionally, proton delivery via bioelectronic platforms does not involve additional solvents or transport matrices and therefore only delivers the substance of interest.

3.5. Further considerations

Aberrant myofibroblast differentiation and continuous expression of collagen type 1 and α-SMA is a common hallmark to many fibrotic diseases [5], some of them being lethal and without any possibility for successful therapeutic intervention. The actual cause of fibrosis in each particular disease is unknown and no effective therapeutic strategy to intervene at an early stage on the molecular level is currently available [6,7]. During healing, an increased metabolic activity takes place in the wound bed, entailing increased proton fluxes for energy generation and hydrogen consumption for molecular assembly to form new tissue [8]. Countering this consumption by the delivery of protons could thereby support wound healing. Importantly, however, in the complex scenario of wound healing and pH, a plethora of mechanisms need addressing, ranging from the effect of pH on extracellular matrix proteins, matrix metalloproteinase activity, and bacterial colonisation [43]. Within this study, one important aspect has been investigated and the promising outcomes on attenuated myofibroblast differentiation at an acidic pH of 6.5 open new avenues for antifibrotic therapy. In stark contrast to the high prevalence of scarring wounds and several investigations regarding the effect of pH during wound healing, underlying mechanisms have not been elucidated to date [8-11]. There is growing evidence that fibrotic disorders, among them wound scarring, are strongly linked to reactive oxygen species (ROS). Therefore, targeting elevated levels of ROS, by either scavenging the active species or inhibiting ROS producing enzymes, presents an interesting approach [45]. In fibrosis, hydrogen peroxide (H₂O₂) is the main source of ROS production and strongly related to the active function of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4) whose sole role is the production of H₂O₂ [46,47]. Upstream in the signaling cascade, the protein SMAD3 has been identified to trigger NOX4 expression and is directly activated by phosphorylation in response to TGFβ1 – TGFβ1 receptor binding [48]. The production of H₂O₂ and related ROS has been suggested to be of major importance in inducing fibroblast to myofibroblast differentiation. H₂O₂ is a small molecule involved in a multitude of mechanisms in cell homeostasis and can easily diffuse through the cell membrane. Its decomposition into other ROS (*OOH, *OH) has been suggested to induce the malignant effect and oxidative stress that result in the fibrotic response. It remains, however, open to what extent acidic wound dressings in general, or the local pH changes reported in this work, interfere with this complex pathway. This decomposition of H₂O₂ into ROS is slowed down under acidic conditions, yet catalysed under alkaline ones. We therefore hypothesise that a low pH will stabilise this decomposition, thereby reducing the formation of ROS and consequently inhibiting fibroblast to myofibroblast differentiation. We note that modern medicine lacks wound healing therapies that are able to address specific aspects of the complex and dynamic wound environment [13,15]. Technologies currently being developed within the field of bioelectronics have the potential to offer several advantages over today’s conventional approaches [26,49]. Namely, the possibility for sophisticated and miniaturised devices that enable precise spatial and temporal control over drug delivery. To-date, bioelectronics technologies have primarily been developed for biological signalling and sensing applications. Thus, from a drug delivery perspective, the use of electronic ion pump technology is not limited to proton transport. In previous work, the suitability of electronic ion pumps for delivering molecular ions has been demonstrated for substances such as small linear ions (e.g., GABA [28]), larger, more rigid compounds based on cyclic or aromatic structures (e.g., auxin [31]), as well as dyes (indigo carmine and methylene blue [36]), indicating...
that continued research on pharmaceutically relevant ion delivery via electronic ion pump technology is ongoing. Remarkably, the delivery and regulation of protons – as the smallest possible ion – in biological systems has received only limited attention. While excellent work has been published on proton delivery systems, including closed-loop feedback systems, no translation from proof of concept models to in vitro studies with investigations into the effect of pH on cell fate has been performed [25,28,44,50–52]. Given the central role of protons in virtually all biological processes, this is an unmet potential for future work in the field of bioelectronics. Delivered by electronic ion pumps in particular, protons can reach their target at the site of interest. However, high buffer capacity of wound fluid or blood is a significant challenge that must be overcome for clinical translation. Ion delivery to biological conditions might be addressed by ion pumps with multiple outlets that provide proton delivery over a larger spatial distribution, simultaneously with highly localised control over total proton concentration. Additionally, a recently developed hybrid proton pumping technology offers a promising solution to this challenge. Strakosas and colleagues delivered high concentrations of protons to buffered solutions (HEPES) by use of a multiarray system based on palladium [51]. Connected via an ion conductive membrane, protons could be delivered from a reservoir to the target and induced significant changes in pH.
While higher delivery efficiency and shorter pumping time might be desired, the buffered system does also reduce a supposedly cytotoxic effect on NHDF that are in direct proximity with the ion pump’s outlet, and would thus experience a too low pH. Over 32 h, the buffering capacity of the culture media, proton pumping, and proton diffusion seem to be in an equilibrium that did not detrimentally affect fibroblasts. Such a scenario could also be envisaged when evaluated in vivo. Furthermore, the pH across the wound and its spatial distribution within wounds of different sizes needs to be understood and addressed [8,9]. In contrast to acidic wound dressings, wound bandages based on electronic ion pump technology could be engineered to deliver protons at defined temporal and spatial resolution. To achieve such a system, further research is needed to address the above-mentioned challenges and better understand the underlying mechanisms of pH attenuated myofibroblast differentiation. The results presented here, based on an established in vitro setting with control over delivered ions, can provide a platform to investigate these scenarios and allow to increase the complexity of in vitro studies, e.g., using coculture systems of fibroblasts and keratinocytes, to further exploit the potential of our system. Furthermore, the technology can be expanded towards the delivery and evaluation of larger ions, for example new candidates of antifibrotic drugs. Such experimental conditions would further shed light on whether the proof of concept presented here could be translated to the in vivo situation. From a technical point of view, proton or drug delivery based on polyelectrolyte IEM systems is not restricted to the syringe-design form factor used in these studies, and large addressable arrays of IEM based devices have been developed [26,53]. Such multiple addressable electronic ion-pump-based devices on flexible and/or stretchable bandages or scaffolds could be integrated with onboard sensing capabilities [21,54–56]. Real-time pH sensing has been acknowledged as an important tool to monitor wound healing and new methods based on e.g. luminescence and chromogenic fibres have been investigated [57,58]. By this, the demand for larger area conformal coverage in wounds, coupled with highly sophisticated electronically regulated wound treatment regimens could be met. Such smart dressings could dynamically change their drug delivery profile to follow the wound healing progression, thus maximising drug efficacy while minimising extraneous drug interactions systemically. We thus envisage high potential for further development of our system to various architectures and the transport of small-molecule drugs in wound healing models or fibrotic scenarios.

4. Conclusion

We could demonstrate that fibroblast to myofibroblast differentiation can be steered by controlling the environmental pH to 6.5. This result was achieved by changing the pH locally with a liquid-flow free electronic delivery device, the electronic ion pump, and verified by manually changing the pH. Gene as well as protein expression of α-SMA and collagen type 1 were significantly reduced in fibroblasts cultured under acidic conditions, compared to differentiated myofibroblasts. We thus conclude that pH should be further addressed in wound healing scenarios and that it has a major contribution to fibrosis on the cellular level. The electronic ion pump provides an ideal foundational tool for these investigations with its versatility in defining local regions and time periods of shifted pH, and the ability to run multiple electronic ion pumps in parallel. Indeed, this setup is first described in this study and represents a new in vitro platform for investigating wound healing scenarios, which can easily be expanded with increasing device sophistication and with additional pharmaceutical or therapeutic compounds. One development pathway aims at exploring areal electronic ion pump matrix systems to generate dynamic pH gradients, combined with sensors, that would allow for a pixelated and autoregulated wound healing technology.

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Author credit statement

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Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] N.J. Percival, Classification of wounds and their management, Surgery (Oxford) 20 (5) (2002) 134, doi:10.1383/surg.20.5.14626.
[2] M.C. Ferreira, P. Tuma Jr., V.F. Carvalho, F. Kamamoto, Complex wounds, Clinics (Sao Paulo) 61 (6) (2006) 571, doi:10.1590/S0102-71532006000600014.
[3] B. Sund, A.K. Arrow, New developments in wound care, Clinica Reports 2000.
[4] A. Goel, P. Shrivastava, Post-burn scars and scar contractures, Int J Plast. Surgery 43 (Suppl) (2010) S63, doi:10.4037/0970-0358.70724.
[5] M.P. Czubryt, Common threads in cardiac fibrosis, infarct scar formation, and wound healing, Fibrogen. Tissue Rep. 5 (2012) 19, doi:10.1888/1755-152X-5-19.
[6] P.S. Tsou, A.J. Haak, D. Khanna, R.R. Neubig, Cellular mechanisms of tissue fibrosis. 8. Current and future drug targets in fibrosis: focus on Rho GTPase-regulated gene transcription, Am. J. Physiol. Cell Physiol. 307 (1) (2014) C2, doi:10.1152/ajpcell.00060.2014.
[7] J. Rosenboom, F.A. Mendoza, S.A. Jimenez, Strategies for anti-fibrotic therapies, Biochim. et Biophys. Acta (BBA) - Molecular Basis Disease 1832 (7) (2013) 1088, doi:10.1016/j.bbadis.2012.12.007.
[8] T. Sirkka, J. Skiba, S. Apeel, Wound pH depends on actual wound size (2016) https://doi.org/10.1371/journal.pone.01606365.
[9] G. Gerth, The significance of surface pH in chronic wounds, Wounds UK 3 (3) (2007) 52.
[10] S.L. Percival, S. McCarty, J.A. Hunt, E.J. Woods, The effects of pH on wound healing, biofilms, and antimicrobial efficacy. Wound Repair Regen. 22 (2) (2014) 174, doi:10.1111/wrr.12125.
[11] L.A. Schneider, A. Kerber, S. Grabebe, J. Dissemond, Influence of pH on wound-healing: a new perspective for wound-therapy? Arch. Dermatol. Res. 298 (9) (2007) 413, doi:10.1007/s00403-006-0713-4.
[12] M.W. Tibbitt, J.E. Dahlman, R. Langer, Emerging frontiers in drug delivery. J. Am. Chem. Soc. 138 (3) (2016) 704, doi:10.1021/jacs.5b09974.
