Expansion of Fibroblast Cell Sheets Using a Modified MEEK Micrografting Technique for Wound Healing Applications

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Abstract

One of many challenges in wound treatment is to provide sufficient wound area coverage to protect against pathogens and speed up the wound healing process. Recently, cell sheet engineering has shown promise in treating deep wounds. Unfortunately, the cell sheet size is too small for practical use. To overcome this limitation, the MEEK micrografting technique, currently being used in the treatment of extensive deep burns, was investigated. This technique was modified and applied to enlarge dermal fibroblast cell sheets, constructed using temperature-responsive PNIAM-co-AM graft copolymer, by transforming the intact cell sheets into small cell islands at expansion ratios of 1:3, 1:6 and 1:9. Afterwards, cell mechanisms essential for wound healing, including reattachment, proliferation, and migration were investigated. The fibroblast cells on MEEK gauzes possessed high cell viability, capable of reattachment and migration. The optimal expansion ratio having the highest migration rate was 1:6, possibly due to an effective intercellular communication distance. Therefore, the combination of cell sheet engineering with the MEEK micrografting technique could provide high quality cells with a large coverage area, which would be particularly beneficial in wound care applications.

Introduction

Human skin is the integument organ that performs vital roles, including a barrier against pathogens, thermoregulation and nerve sensation. Thus, lacking skin or suffering a skin injury gives rise to many complications, including infection, tissue necrosis, hematomas, and etc. Although some types of wounds or injuries repair themselves quickly, on occasion the healing can be protracted. Prolonging the healing time can increase the risks or damage when skin is lost. In recent years, scaffold-free cell sheet engineering has been shown to be a promising treatment for chronic wounds or harmful injuries by providing a high re re-epithelialization rate and reducing wound closure time[1,2]. However, the cost of cell sheet construction is expensive due to the manufacturing process of PNIAM grafted surfaces, commercially available under the tradename "UpCell™." Moreover, the cell sheet coverage is also restricted, depending on the culture dish shapes and sizes, the largest available is a 100 mm circular dish. In the previous work, we had developed an in-house method to fabricate temperature-responsive surfaces for cell sheet construction using PNIAM-*co*-AM graft-copolymer by UV irradiation[3,4]. The cell sheets fabricated by PNIAM-*co*-AM coated plates possessed the physical and biological properties similar to those constructed by using UpCell™ plates including the cell viability, the size of the harvested sheets and the protein expression[5]. Besides significant cost reduction in the cultureware, by using the co-polymer method, it allowed us to construct various shapes and sizes of cell sheets. Unfortunately, the size of the harvested cell sheet was still not adequate to cover a typical wound area, being approximately ten times larger than the current harvested cell sheets [6,7]. Using larger tissue culture dishes could overcome this problem. However, a significantly large number of cells are required for the cell sheet construction, leading to a long period of cell expansion, so increasing the risks and complications during hospitalization. When the cell sheets are detached from the temperature-responsive culture surfaces, they undergo cytoskeletal re-organization[8,9], causing the cell sheet shrinkage resulting in practically a 40% size reduction [5].

Therefore, increasing the temperature-responsive culture surface area may not be the fabrication solution to fabricate sufficiently large cell sheets required for clinical treatment.

In the treatment of large burn wounds, skin graft meshing is often used to increase the area of the skin graft and also allows exudate to escape. However, the mesh with expansion ratios above 1:6 was shown to be impractical, delaying the re-epithelialization process[10]. Due to the limited expansion ratio of skin graft meshing, a new technique, called MEEK Micrografting, was introduced[11, 12]. This technique can enlarge the original skin graft (42 Î 42 mm^2) by several expansion ratios: 1:3, 1:4, 1:6 and 1:9. To enlarge the skin graft, a split-thickness skin graft is collected from an available donor site. After that, the harvested skin graft is placed on a 42 Î 42 mm moistened cork plate epidermis side up, and the skin is cut to the same shape and size of the cork plate. Next, the cork plate with the skin graft is placed onto a cutting block to fix the position of the sample and passed through a cutting machine, containing 13 parallel blades with 3 mm spaces. The cork plate is rotated 90 degrees before passing through the machine to create 196 small square islands. Subsequently, the sample is carefully removed from the cutting machine and the top of the skin graft is sprayed with adhesive dressing and rested for 3–5 min to allow the glue to set. Afterwards, the epidermis coated with the adhesive glue is placed onto pre-folded gauze, and the cork plate is removed, transferring the skin islands to the pre-fold MEEK gauze. All four sides of the gauze are then unpleated to enlarge the skin graft area. The aluminum foil underneath the gauze is peeled off before transplanting the enlarged skin graft on the patient's wound. The expanded skin grafts at the ratios of 1:3, 1:4, 1:6 and 1:9 result in the areas of 73 Î 73 mm^2, 84 Î 84 mm^2, 102 Î 102 mm^2, and 126 Î 126 mm^2, respectively, and the spaces between each graft island are 2.20 mm, 3 mm, 4.35 mm and 6 mm, respectively [13–15]. The benefits of the MEEK Micrograft method include a higher skin graft expansion ratio, more accurate portion of expansion than traditional mesh grafting, and ease of handling [11,16]. The MEEK expansion ratios of 1:4–1:9 are predominantly used to treat severe burn injury, providing a high re-epithelialization rate in patients either with or without diabetic conditions in addition to reducing mortality and hospitalization [10,16–18]. Also, patients who received MEEK treatment have statistically low evidence of graft rejection or contamination because the skin graft lacks the skin tissue connection between skin lands [12,13].

Based on these benefits, we proposed to apply a modified MEEK micrografting technique to expand the harvested skin cell sheets constructed from the temperature-responsive polymer surfaces, which, to the best of our knowledge, has never been tested before. In this study, the harvested human fibroblast cell sheets were expanded at expansion ratios of 1:3, 1:6 and 1:9 by using a modified MEEK technique. The characteristics of the cell sheets after being expanded, including cell viability, reattachment capability, growth potential and migration capacity were compared with the cell sheets on typical nylon wound dressing, which was made of a polyamide material similar to the gauzes used in the MEEK micrografting technique and served as the control.

Results

Cell viability and reattachment of the fibroblast sheet on nylon dressing

Since a monolayer fibroblast sheet is fragile and can be folded easily, the possibility of using nylon dressing as the support to transfer the cell sheet and place it on a wound was explored. Nylon dressing is a good candidate for this application, as it has already been routinely used as a wound dressing material. When dry nylon dressing was placed on top of the detached fibroblast cell sheet, the whole cell sheet immediately attached to the gauze with no further shrinkage (Fig. 1c). The fibroblast cells were allowed to grow on the nylon dressing for seven days to determine whether the nylon material could support the growth of fibroblasts. According to Fig. 2, confluent cells with spindle morphology were observed outside the yellow dashed line, which represents the initial edge of the cell sheet, and the arrows indicated the direction of the outgrowth cells. This evidence suggests the fibroblast outgrowth from the cell sheet into the adjacent nylon dressing area. In addition, the cell viability of the outgrowth cells remained high, close to 100%, throughout the experiment, indicating that the cells were healthy and active. Moreover, the reattachment ability of the fibroblast cell sheets from the nylon dressing adhering to a tissue culture surface was also analyzed. As shown in Fig. 2, very few fibroblast cells, approximately 1% confluency, was found to migrate from the nylon dressing initially, but after seven days the number of the reattached cells continuously increased to over 60%, having high cell viability and correct morphology. Interestingly, on day 7, most of the reattached cells migrated out of the sheet at the edge, in which the red arrows show the edge of the cell sheet before the dressing being removal. Hence, the nylon dressing could be used as a structural support for cell attachment, growth, and migration.

Cell viability after applied MEEK method

Based on the MEEK technique, the samples must be cut and stretched to create numerous 3Î3 square cell islands. Applying this technique to the cell sheets may cause damage to the cells, affecting the cell migration and proliferation. Therefore, the viability of fibroblasts on MEEK gauze after being expanded was examined by using a LIVE/DEAD staining kit, in which green fluorescence indicated live cells, while red fluorescence indicated dead cells, and their intensities were quantified over 7 days. Figure 3 shows the intense green fluorescent cells inside the yellow boundary which indicated the presence of the cell sheet islands, while the more dispersed green cells were observed outside the yellow boundary, representing the outgrowth areas. The viability of the fibroblast cells was high at all time points, regardless of the cutting and stretching process. These fibroblast cells on MEEK gauze migrated and proliferated outside the cell sheet island and occupied in the surrounding areas. As a result, MEEK gauze has also been shown to support the fibroblast migration and proliferation.

Cell reattachment of cell sheet on MEEK 1:3, 1:6 and 1:9 expansion ratio

Following the MEEK micrografting technique, the skin islands on MEEK gauze are normally transferred and attached to the wound, and those transferred skins facilitate re-epithelialization. To prove that this concept was also true for the cell sheets on MEEK gauze, we investigated the ability of the fibroblast cells on MEEK gauze to translocate and re-attach to a new culture dish surface. Within 3 days, the cells migrated from the cell sheet islands and reattached onto the new culture surface, and those cells continuously proliferated. As shown in Fig. 4a, on day 7, many more cells were found on the surface and had spread to most areas. Clearly, the cells migrated from the edge of the previously occupied cell sheet islands, indicated by the red arrows. On day 14, the reattached fibroblast cells became more confluent and denser, enclosing the empty spaces. On day 21, the cells grew and migrated through the inside sheet island area, providing the whole culture surface coverage. The cell confluency were determined and are shown in Fig. 4b. The average cell confluency was initially lower than 20%, but continuously increased to reach to 100% by day 21. However, the confluences between different expansion ratios were not statistically significant. This result shows that MEEK gauzes could allow the cell reattachment and enable prolonged cell cultivation, providing high confluency within 21 days.

Cell migration of fibroblast sheet on the nylon dressing and MEEK gauze at 1:3, 1:6 and 1:9 expansion ratios

The cell migration on nylon dressing and MEEK gauzes was investigated, as it was the fundamental process of tissue homeostasis and wound healing. The fibroblast migration patterns on the nylon dressing, and MEEK gauzes at expansion ratios of 1:3, 1:6 and 1:9 are shown in Fig. 5; where the dotted lines indicate the initial edge of the cell sheets on the nylon dressing, or the cell sheet islands on MEEK gauzes. Since the cell sheets on the MEEK gauzes were cut into smaller islands, clear straight-line edges with dense cells were observed. Unlike the MEEK technique, the cell sheet was directly placed onto the nylon dressing, showing a gradient of cell density around the periphery. Consequently, the cells on the nylon dressing could migrate along the fiber towards less crowded areas with elongated morphology immediately, as shown in Fig. 5 and Supplementary S1. On the MEEK gauze, the fibroblasts mostly remained stationary during the first 24 h but moved forward from the periphery of the cell sheet islands along the fiber of the MEEK's polyamide gauze, with the cells on the 1:6 MEEK gauze having the furthest migratory distance (Fig. 5 and Supplementary S2).

Comparison of the average velocities and trajectories of the cells on nylon dressing and MEEK gauzes

The average velocities of the cells on nylon dressing and MEEK gauzes at expansion ratios of 1:3, 1:6, and 1:9 at various time points are shown in Fig. 6. The mean non-overlapping velocities of the cells at 6 h were low and similar in all groups. During 12–48 h, the average velocities of the cells on the MEEK gauze at the expansion ratio of 1:6 were the highest. During 60–72 h, the average velocities of the cells on the nylon dressing remained constant, while those of the cells in other conditions noticeably increased. The migratory paths of the fibroblasts on all gauzes were tracked and shown as wind-rose plots in Fig. 7. Each color line represents the total path of a single fibroblast over 72 hours. As shown in Fig. 7, the larger diameters of the wind rose plots were observed from the fibroblast cells at the interior and exterior on the

MEEK gauze at 1:6 ratio, indicating the enhanced motility of these cells at this expansion ratio. The fibroblast cells on other surfaces had noticeably shorter migratory paths.

Discussion

The major obstacle to using skin cell sheets in clinical practices is their size limit[5]. In real applications, the skin cell sheets need to be at least ten times larger than their current size for full wound area coverage to promote effective treatment[6,7]. To overcome this issue, we first considered constructing a larger cell sheet by using a larger surface area, because the area of the temperature-responsive culture surface directly affects the size of the harvested cell sheets. However, with an increased temperature-responsive culture surface size, a significantly large number of cells were required to obtain the same seeding density, which was very labor intensive and prone to technical errors. We, therefore, modified our protocol by reducing the cell density and allowing the cells to proliferate for a few days until confluence. Unfortunately, by increasing the cell culture time on the temperature-responsive polymer, the cells attached too strongly to the surface, making detaching the cells as an intact sheet challenging. During the process of cell sheet harvesting, the cell sheet was broken into smaller (Supplementary S3). Thus, we proposed the use of nylon dressing or MEEK gauzes as cell sheet supporting material during cell sheet harvesting and for cell sheet expansion. Nylon is an inexpensive material and currently used as wound dressing, while MEEK pre-folded gauze is currently used in severe burn patients, giving a high re-epithelialization rate [12,17,19]. To verify their potential application with cell sheet engineering, we investigated the qualities and behavior of fibroblast cell sheet on both fabrics.

Our results showed that the cell sheets could firmly attach to the nylon dressing and MEEK gauzes with no adhesives (Figs. 3 and 4). More importantly, the fibroblast cell sheets on the MEEK gauzes did not detach from the gauze, even after being cut and stretched. The strong attachment of the cell sheets to both materials was possibly due to the adhesive protein that was harvested together with the cell sheets[20]. Normally, in the MEEK technique for skin grafts, the proprietary glue, Humeca® glue, is required to keep the donor skin sample in place during the cutting process and is critical to the outcome of the skin graft transfer[21]. Fortunately, with the adhesive protein harvested with the cell sheet, Humeca® glue was unnecessary. This would be particularly beneficial in reducing the preparation steps in the protocol and there would be no concern over the effect of the synthetic adhesive glue on the cell viability and migration.

Besides the cell attachment, both the nylon and MEEK fabrics were biocompatible with the cells and could clearly support the cell growth. The fibroblast cells on the nylon support grew well for over 7 days, and the outgrowth cells were healthy. For the MEEK samples, we only evaluated the cell viability at the expansion ratio of 1:9, as a representative for all the expansion ratio. The cell viability was found to be over 95% and the cells at the edge migrated and proliferated into healthy cells. Hence, we can assume that even though cutting the cell sheets might have caused the cell damage around the edges, it did not have any effects on the cell proliferation and migration. According to our previous study, the fibroblast

cell sheets could help accelerate the wound healing process by releasing essential cytokines and growth factors that regulated the wound repair[5]. A higher cell number would lead to more cytokine and growth factor secretion, possibly leading to faster wound healing.

The migration patterns between the fibroblasts on the nylon dressing and MEEK gauze were quite similar, as the cells migrated from the periphery of the cell sheets and preferentially moved along the matrix fibers (Fig. 5). The aligned fibers could induce cellular elongation and the alignment of collagen secretion by fibroblast, guiding the migration of the cells to the defect area [22,23]. According to the migration movie clips (Supplementary S2), individual fibroblasts initially detach themselves from the monolayer and moved away in a non-coherent pattern, but directed towards free space. The migration of fibroblasts occurring earlier on nylon dressing, as compared to MEEK gauze, was possibly the result of the lower cell density at the periphery of the cell sheets. At a lower cell density, stronger cell-substrate interactions overcome cell-cell interactions, allowing the cells to escape the monolayer[24]. On the other hand, in all MEEK conditions, single cells took longer to detach from the dense cells at the edge of the monolayer due to strong cell-cell interactions, leading to immediate net movement at earlier time points.

Our result showed that the migration rates of fibroblasts on the MEEK gauzes were predominantly higher than on the nylon dressing. Even though both fabrics were made of the same polymer, which is polyamide, the topography of the substrates and densities of the fibers were clearly different. These factors have been previously shown to affect the cell-substrate adhesion properties[25], which directly influences the cell motility[26]. In addition, cutting the cell sheets into small cell sheet islands in the modified MEEK technique resembled wounding the cell monolayer in a scratch assay, which was reported to affect the cell migration by inducing changes in gene expression and signaling[27]. There was the evidence that cells produced chemicals or signals, such as ATP or Ca^{2+} after injury[28,29]. Increases in the level of ATP and Ca^{2+} have been shown to enhance and stimulate fibroblast proliferation, migration, and ECM productions which are involved in wound healing mechanisms[28]. Another possible explanation for a higher migration rate of fibroblast cells on the MEEK gauze is the mechanical stimulation due to the stretching of the MEEK gauze for the cell sheet expansion. Stretched fibroblasts have been reported to migrate faster and move a further distance, as compared to their non-stretched counterparts[30]. Stretching caused the up-regulation of matrix metalloproteinases (MMPs), which were responsible for collagen degradation, leading to lower cell-substrate interaction and resulting in the increased migration[30].

When comparing between different expansion ratios in the MEEK technique, the migration rate of fibroblasts on the MEEK gauze at 1:9 expansion ratio was lower than that of the cells on the MEEK gauze at 1:6 expansion ratio, which could have resulted from a greater distance between the cell sheet islands. Generally, cells communicated with each other through the release of soluble cytokines and chemokines. These signaling molecules diffuse through the medium, bind to the cell's receptors and activate many crucial biological pathways responsible for proliferation, migration, etc[31]. The longer distance between each cell island on the MEEK gauze at a 1:9 expansion ratio would have led to larger diffusion length and possibly lower cytokine concentrations to induce the cell migration. Thus, the MEEK at expansion ratio of

1:6 and 1:3 would be more appropriate for wound management, as the cell sheets on MEEK gauzes at these expansion ratios could migrate faster to accelerate wound healing. However, using the expansion ratio of 1:3 may not be significantly beneficial, as it can only cover a small wound area, which commonly heals quite quickly[17]. Considering the cell viability and migration potential, our result suggests the use of MEEK technique at an expansion ratio of 1:6 to expand skin cell sheets to treat large chronic and burn wounds.

In conclusion, we have shown that the combination of the fibroblast cell sheet with nylon dressing or MEEK gauzes at various expansion ratios may overcome the limitation of the cell sheet applications in clinical settings, including limited treatment area and cell sheet handling. Fibroblast cells on nylon dressing and the expanded fibroblast sheets on MEEK gauzes have shown to possess high capacities of cell proliferation and migration which could be particularly beneficial in the wound care applications. This combination technique can be applied to different cell types, such as keratinocytes and endothelial cells to construct large cell sheets and apply to the wounds to speed up the re-epithelialization[32] and vascularization processes[33].

Methods

Dermal human fibroblast cell isolation

The collection of discarded split thickness skin samples and all experimental protocols were approved by the Human Research Protection Unit, Faculty of Medicine, Siriraj Hospital, Mahidol University (Si 587/2017). All methods were carried out in accordance with relevant guidelines and regulations. Fibroblast cells were isolated as previously described [5]. Briefly, a split-thickness skin sample was cut into 1 cm wide strips, and the skin was immersed in 2.4 U/mL of Dispase II (Invitrogen, Carlsbad, CA, USA) overnight at 4^{o}C to disassociate the connective tissue. The epidermis or darker layer was peeled from the skin, leaving only the dermis layer, which was finely chopped and kept in Trypsin/EDTA 0.25% solution (Invitrogen) for 30 min at 37^{o}C. Then, the fibroblast cells were collected by centrifugation at 1500 RPM for 5 min. The cell pellet was resuspended in its culture medium, composed of Dulbecco's Modified Eagle Medium and Ham's F'12 (Invitrogen), at a 3:1 ratio supplemented with 10% fetal bovine serum (FBS) and 1% Antibiotic-Antimycotic. The cells were kept in a 5% CO_2 humidified environment at 37°C.

Fabrication of PNIAM-co-AM grafted plates

The fabrication of PNIAM-*co*-AM grafted surfaces followed the method developed by Sakulae, et al. [4]. In short, 35 mm polystyrene culture dishes (Falcon 3001, BD Bioscience, Billerica, MA, USA) were exposed to UV light (UVGL-58, 6 W) at 254 nm for 30 min to activate the culture dish surface. All the chemicals used in this procedure were purchased from Sigma-Aldrich (St. Louis, MO, USA), except for acrylamide (Merck, Kenilworth, NJ, USA). A mixture of *N*-isopropylacrylamide (NIAM) and acrylamide (AM) at a 1:1 molar ratio, N,*N*'-Methylenebisacrylamide (MBAM), a cross-linker, and potassium periodate (KIO_4), a photo-

initiator, was added and spin-coated onto the UV-activated culture dish at 1500 RPM for 5 min. Then, the surface was immediately exposed to 254 nm UV for 2 h. Afterwards, the polymerized surface was rinsed with 70% ethanol to remove unreacted monomers and dried in a vacuum oven at 30^{o}C for 24 h [3,4]. To sterilize the PNIAM-*co*-AM grafted dishes for cell culture use, these dishes were washed with 70% ethanol, followed by rinsing with phosphate buffered saline (PBS, Invitrogen) three times.

Cell sheet construction using the PNIAM- co -AM grafted plates

The construction of a monolayer cell sheet has already been described elsewhere [5]. Human fibroblast cells at passage 6–9 were seeded onto 35mm of PNIAM-*co*-AM grafted dishes at a density of 3.5×10^5 cells/cm^2, and incubated at 37^{o}C for 24 h to allow the cells to firmly attach to the surface. After that, the incubation temperature was reduced to 10^{o}C for 30 min and later increased to 20^{o}C for 60 min to allow the cells to detach from the surface as an intact cell sheet. The cell sheet was transferred to a new culture dish by pipetting to unfold and straighten out the cell sheet before placing onto a gauze.

Transferring of the cell sheet onto nylon dressing

Nylon dressing (3M™ Tegaderm™ Contact Layer, St. Paul, MN, USA) was cut into circles having the same diameter as a 35 mm culture dish and sterilized by using an autoclave. After the fibroblast cell sheet was harvested and transferred to a new culture dish, dry circular nylon dressing was overlaid on top of the cell sheet and immediately flipped using forceps to allow the cell sheet to face upward (Fig. 1a, c). Five hundred microliters of the fibroblast medium were gently added to the side of the nylon dressing to prevent the cells from drying before the sample was incubated overnight at 37°C. The attachment of the fibroblast cell sheet to the nylon dressing was confirmed using a phase contrast microscope.

Enlargement of cell sheets using the modified MEEK technique

The fibroblast cell sheets were enlarged using a technique modified from MEEK micrografting [12,13]. In this study, only polyamide pleated sheets with aluminum backing or MEEK gauze (Humeca BV, Enschede, The Netherlands) at expansion ratios of 1:3, 1:6 and 1:9 were used. The fibroblast cell sheets were transferred onto MEEK gauzes using the same procedure as that of the nylon dressing. After the cell sheets were well positioned on MEEK gauzes, they were cut vertically and horizontally by hands using a surgical blade, following the previously marked pleats on the gauze. To separate each cell sheet square island, the MEEK gauze was stretched on all four sides, until the gauze became completely unfolded and the aluminum backing was removed (Fig. 1b, d). Next, the cell sheet islands on the MEEK gauze were maintained for 24 h in a small volume of the fibroblast's culture medium to prevent the cell sheets from floating upward.

Cell viability after transferring to nylon dressing and MEEK gauze

The viability of fibroblast cells on the nylon dressing and on the expanded MEEK gauze was analyzed using LIVE/DEAD staining kit (Invitrogen, Carlsbad, CA, USA) over 7 days. At the pre-determined time

points, the cells were stained with a LIVE/DEAD solution, comprising Calcein-AM and ethidium homodimer-1 (EthD-1), according to the manufacturer's instruction. Afterwards, the fluorescence of the stained cells were observed using Cytell™ Cell Imaging System (GE Healthcare, Arlington Heights, IL, USA) at the excitation/emission wavelengths of 485/530 nm and 530/645nm for Calcein-AM and EthD-1, respectively. Its fluorescent intensities were quantified by ImageJ software (NIH, Bethesda, MD, USA).

Transfer of fibroblast cell sheets from nylon dressing and MEEK gauze to new surfaces

The cell sheets on the nylon dressing and MEEK gauzes were turned upside down and placed onto new 35 mm tissue culture dishes to allow the cells to re-attach. Two milliliters of the culture medium were added to these dishes to maintain cell growth. The medium was changed every 2–3 days. At the pre-determined time points, the gauzes were removed from the culture dish before observation using a phase-contrast microscope. In the nylon sample, the translocated cells were investigated on days 3, 5 and 7, while the observation of the cells from MEEK gauze were prolonged to 14 and 21 days. The confluency of the reattached cells was analyzed by ImageJ software in the area where the cells grew out and outside the gauze coverage.

Determination of the cell migration, velocity, and trajectory on nylon dressing and MEEK gauze

To track the cell movement, the fibroblast cells on the gauzes were initially stained with NucBlue™ Live ReadyProbes™ Reagent (Molecular Probes, Waltham, MA, USA) at 20°C for 20 min. Then, the stain solution was removed and 2 µl of fresh medium was added to maintain the cell culture during evaluation. The cell sheet samples on the gauzes were placed on tissue culture dishes with the cell sheets facing down. Afterwards, time-lapse images of the cell migration on the gauze were acquired on Biostation IM-Q microscope (Nikon Inc, Melville, NY) with a 10X magnification (numeric aperture 0.5), and taken every 10 min at various spots for 72 h at 37°C in a 5% CO_2 humidified atmosphere. The determination of the mean non-overlapped velocity and trajectory of the cells were analyzed using CL-Quant software version 3.30 (Nikon Corporation, Minato-ku, Tokyo, Japan).

Statistical analysis

All the results are presented as mean ± SD. A two-way ANOVA followed by Tukey's multiple-comparison tests (GraphPad Software, La Jolla, CA, USA) was applied to analyze the difference between groups of data. The statistically significant difference was considered when the P-value was less than 0.05.

Declarations

Competing interests

The authors declared no competing interests.

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Author contributions

K.B. performed the experiments, analyzed the data and wrote the paper. P.S. and W.S. fabricated PNIAM-*co*-AM grafted surfaces. P.M., K.C. and N.N. assisted in the experimental design with human skin samples. K.V.-P. supervised the research, analyzed the data and revised the paper. All authors read and approved the final manuscript.

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Figures

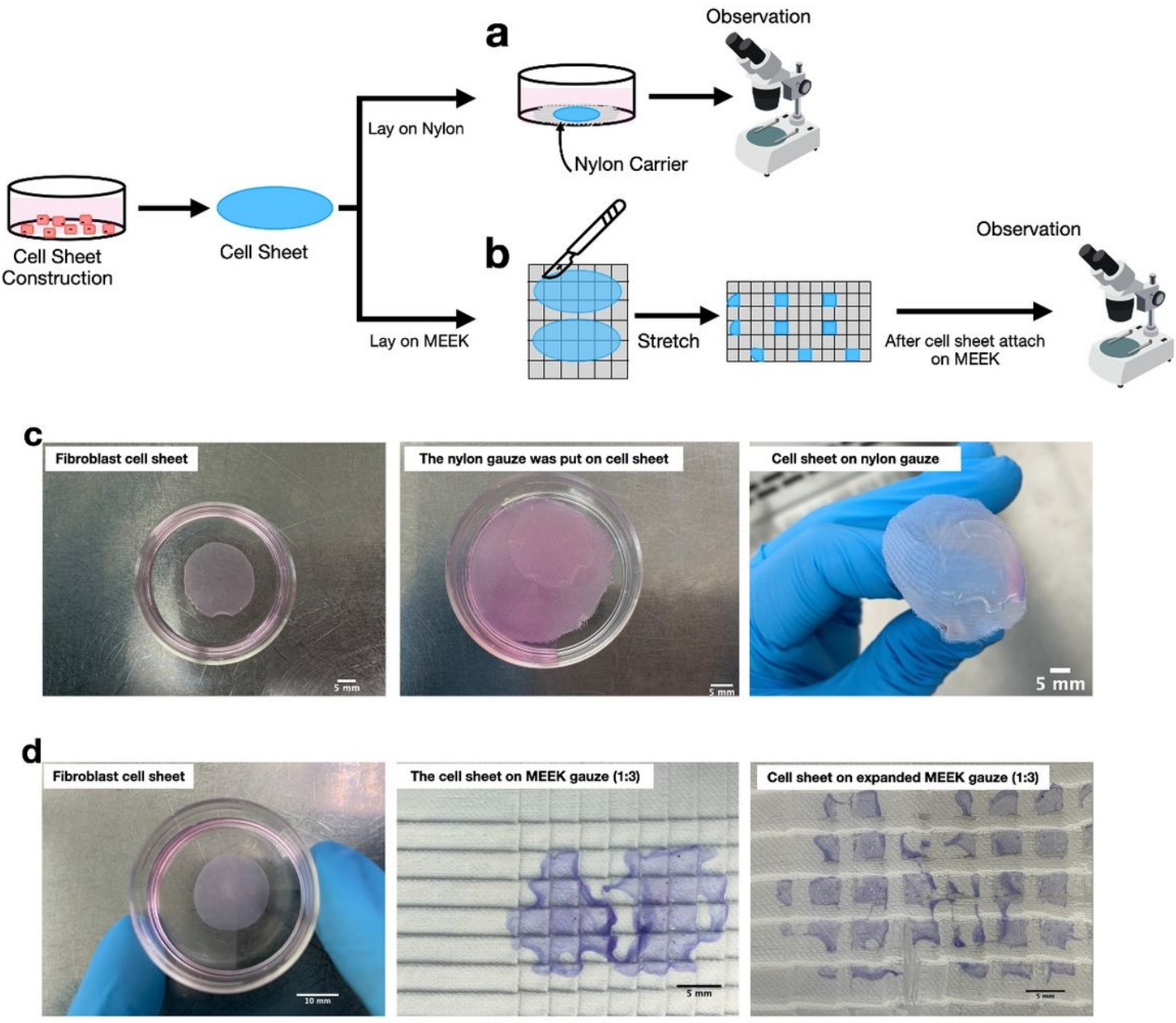


Figure 1

A brief schematic representation of the experimental procedure for transferring fibroblast cell sheets onto nylon dressing and MEEK gauzes. After the cell sheet was harvested from the temperature-responsive culture surface, (a) the sheet was immediately transferred to sterile nylon dressing before observation. Otherwise, (b) the cell sheet was transferred to pre-folded MEEK gauze. Afterwards, the sheet was cut vertically and horizontally using a surgical blade. Then, the MEEK gauze was stretched to all sides to separate the cell sheet square into many small cell islands. (c) The nylon dressing was overlaid on top of the fibroblast sheet before it was flipped using forceps, allowing the cell sheet to face up. (d) The fibroblast sheet was transferred onto the MEEK gauze before being cut and stretched, showing several square cell islands. Note that the cells were stained with typan blue for ease of visualization.

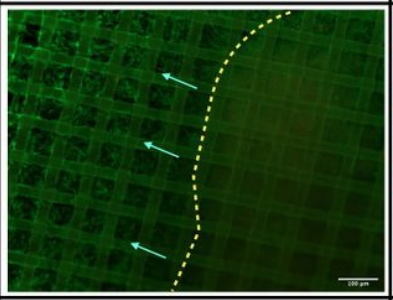
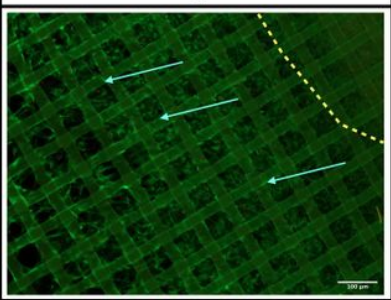
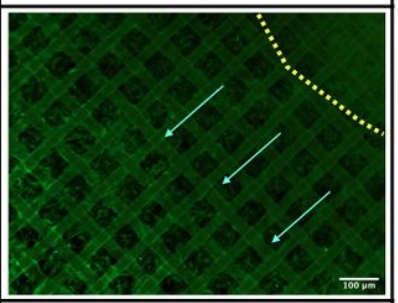
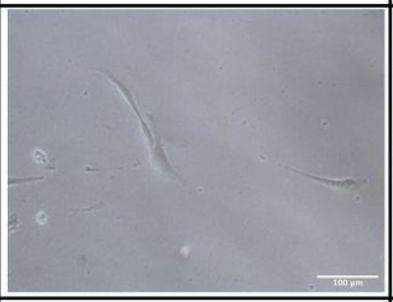
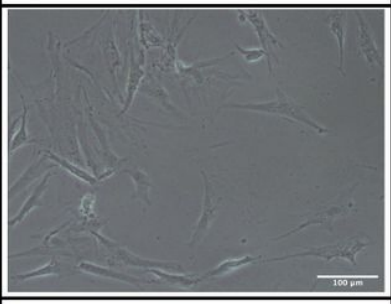
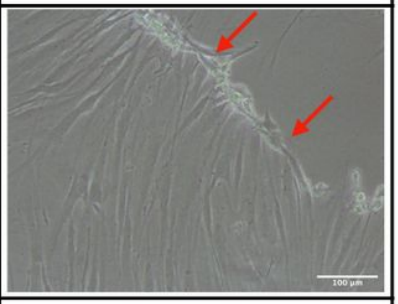
Day	Day 3	Day 5	Day 7
Cell Viability on Nylon			
%Viability of Cell on Nylon	99.97 ± 0.04%	97.83 ± 1.76%	98.93 ± 0.78%
Reattached Cells			
%Viability of Reattached Cells	99.88 ± 0.06%	99.82 ± 0.07%	99.92 ± 0.08%
% Cell Confluency	1.26 ± 1.12%	21.49 ± 8.06%	63.20 ± 18.50%

Figure 2

Viability of the fibroblast cell sheets on nylon dressing and the cell reattachment. (A) The fluorescence images of LIVE/DEAD stained fibroblast cells on nylon dressing at day 3, 5 and 7 and their cell viability percentages were presented. Note that the green color fluorescence represents live cells, while red fluorescence indicated dead cells. (B) The phase-contrast images show the morphology of the fibroblasts that are re-located to a new culture dish surface at various time points. The confluency and viability percentages of the re-attached fibroblast cells were calculated and shown in this figure. The yellow dotted line represented the edge of the fibroblast cell sheet, and the light blue arrows indicated the outgrowth cell direction on the nylon dressing. The red arrows showed the area that the cell grew out from the edge of sheet. Results are displayed as mean ± SD (n = 6). The scale bars represent 100 mm.

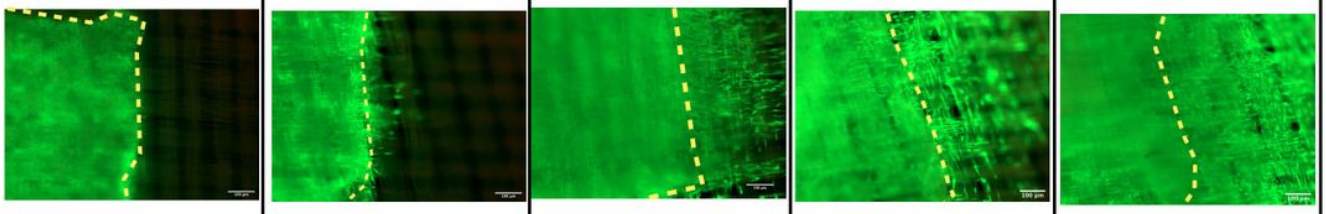
	Day 0	Day 1	Day 3	Day 5	Day 7
Cell Viability on MEEK					
%Cell Viability	99.01 ± 0.73 %	98.06 ± 1.17 %	98.13 ± 1.33 %	96.62 ± 3.10 %	96.65 ± 2.70 %

Figure 3

Cell viability of fibroblasts on 1:9 MEEK gauze. The fibroblast cell islands were stained with LIVE/DEAD stain on day 0, 1, 3, 5 and 7, in which green and red fluorescence refer to live and dead cells, respectively. The cell viability was determined from an image analysis (n = 3). The yellow dotted lines indicate the edge of the fibroblast sheet island. Results are displayed as mean ± SD (n = 10). The scale bars represent 100 mm.

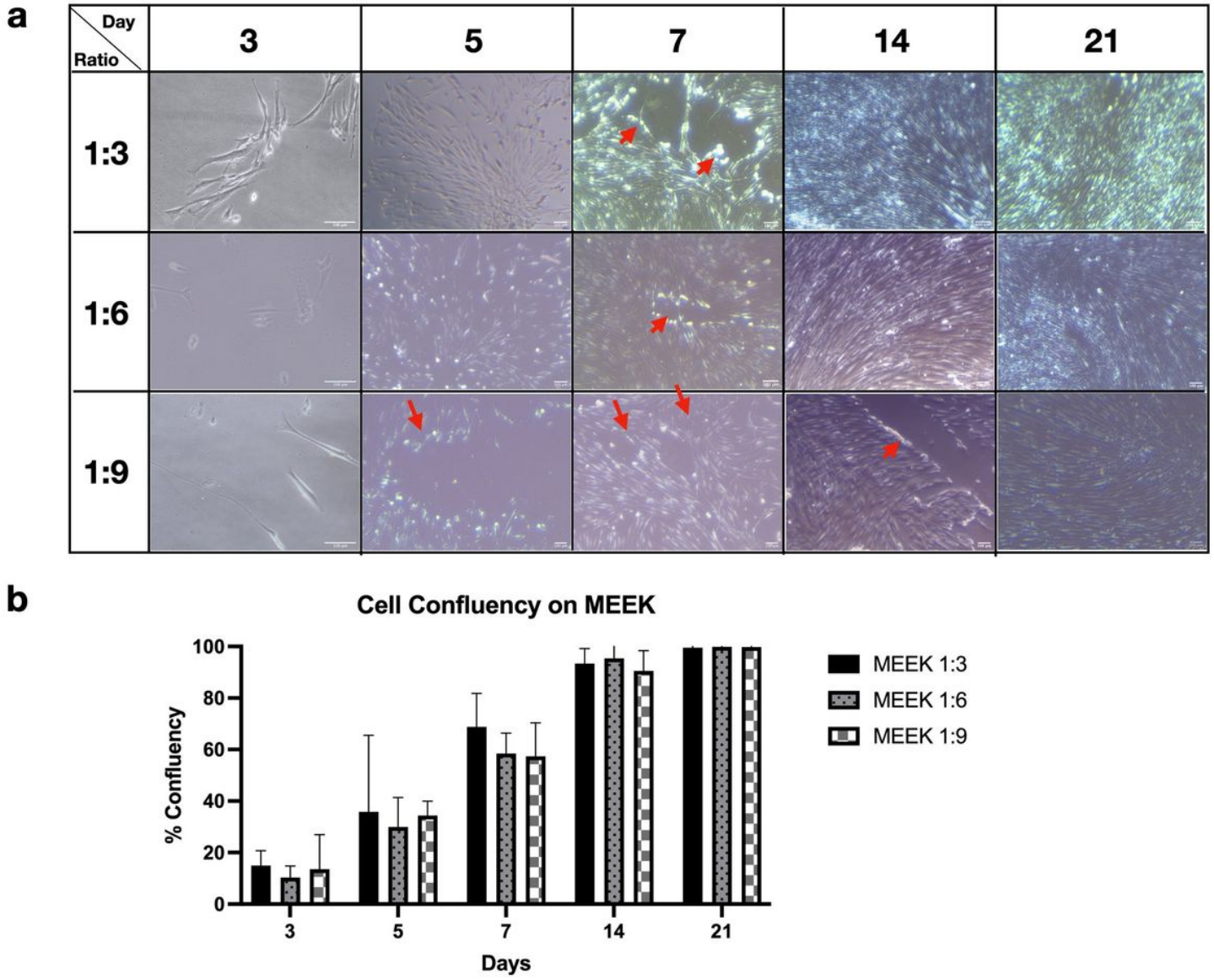


Figure 4

Reattachment of fibroblast cells from MEEK gauze to new culture dishes. (a) The presence of fibroblast cells from MEEK gauzes at 1:3, 1:6 and 1:9 expansion ratios were observed at day 3, 5, 7, 14 and 21 under a phase contrast microscope. The red arrows indicate the edge of the cell sheet islands. (b) The confluency of fibroblasts on new surfaces at various time points were determined at various time points. Results are displayed as mean ± SD (n = 6). The scale bars represent 100 mm.

Figure 5

Fibroblast migration on both nylon dressing and MEEK gauzes. The fibroblast movement was shown at 0, 24, 48 and 72 h on nylon dressing and MEEK gauzes at 1:3, 1:6 and 1:9 expansion ratios. The dotted

lines indicate the initial edge of the cell sheet or cell sheet island. The scale bars represent 100 mm.

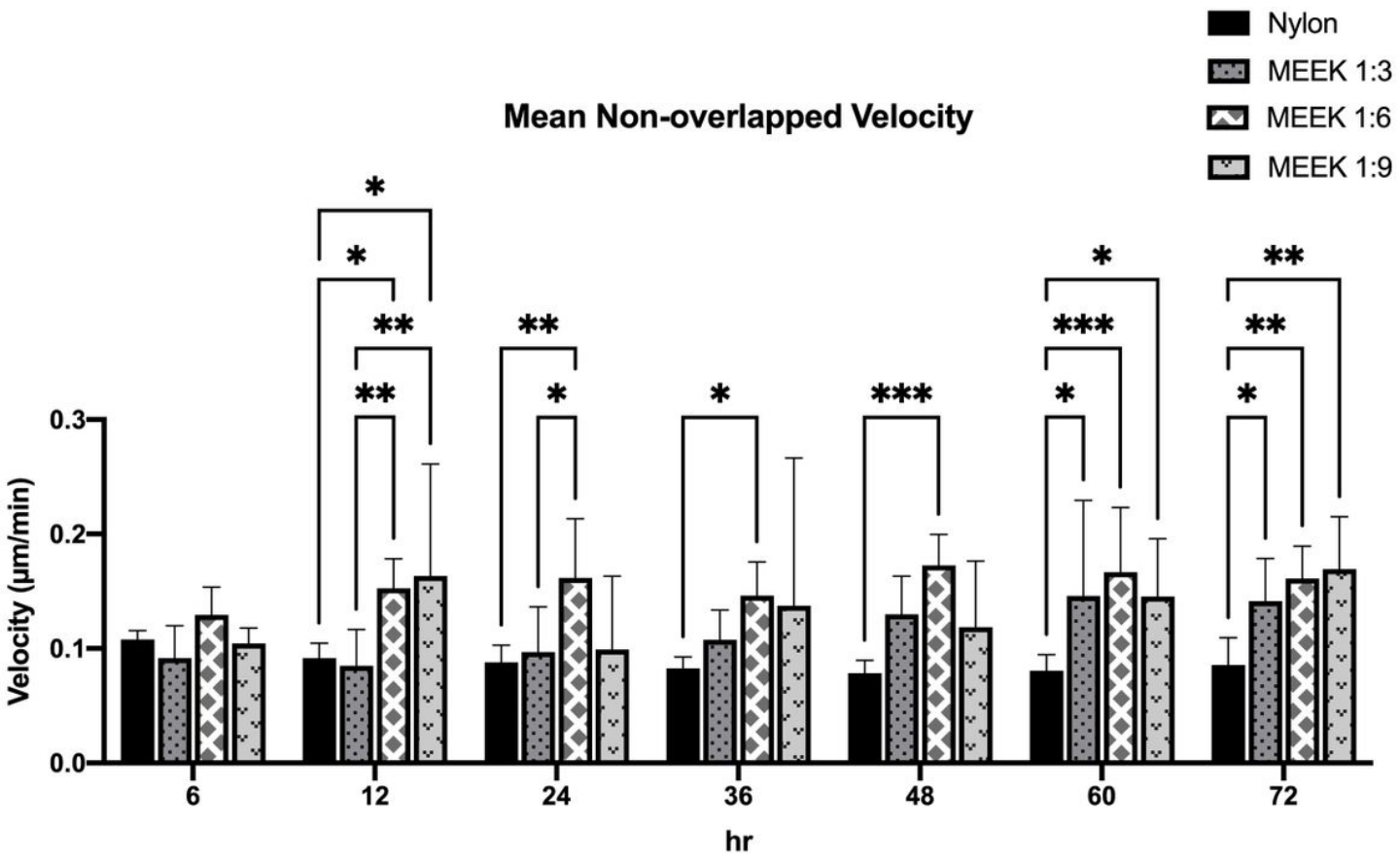


Figure 6

Mean non-overlapped velocity of fibroblast cells on nylon dressing and MEEK gauzes. The migration velocities of fibroblasts on nylon dressing and MEEK gauzes at 1:3, 1:6 and 1:9 expansion ratios were determined at 6, 12, 24, 36, 48, 60 and 72 h. Results are shown as mean ± SD (n = 5). Statistically significant difference was determined using two-way ANOVA with Tukey's multiple comparisons test, in which *p < 0.05; ** p < 0.01, and ***p < 0.001.

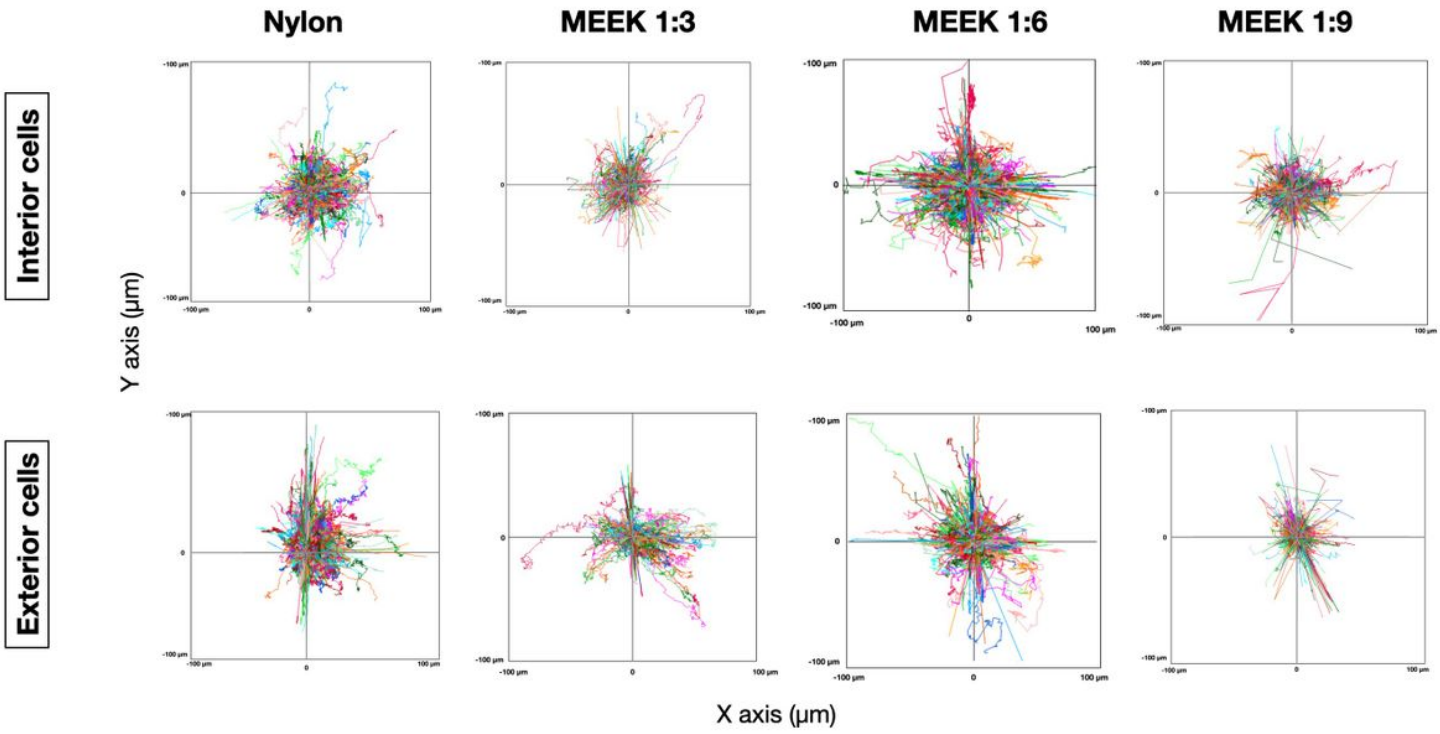


Figure 7

Wind-Rose plots depicting the migratory behaviors of fibroblast cells on various materials. The fibroblast cells on nylon dressing and MEEK gauzes at 1:3, 1:6 and 1:9 expansion ratios were stained to track their movement, and the cell trajectory was analyzed in each condition. The trajectory plot represents cell migration direction and distance at the interior and exterior of both nylon dressing and MEEK gauzes during 0 – 72 h. Each line indicates an individual path of each cell.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MEEK13new.mov
- MEEK16.m4v
- MEEK19.m4v
- NylonReDAPI.m4v
- SupplementaryInformation.docx