Research article

In-process monitoring of a tissue-engineered oral mucosa fabricated on a micropatterned collagen scaffold: use of optical coherence tomography for quality control

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ABSTRACT

Background: We previously reported a novel technique for fabricating dermo-epidermal junction (DEJ)-like micropatterned collagen scaffolds to manufacture an ex vivo produced oral mucosa equivalent (EVPOME) for clinical translation; however, more biomimetic micropatterns are required to promote oral keratinocyte-based tissue engineering/regenerative medicine. In addition, in-process monitoring for quality control of tissue-engineered products is key to successful clinical outcomes. This study aimed to update our technique to fabricate a more biomimetic DEJ structure of oral mucosa and to investigate the efficacy of optical coherence tomography (OCT) in combination with deep learning for non-invasive EVPOME monitoring.

Methods: A picosecond laser-textured microstructure mimicking DEJ on stainless steel was used as a negative mould to fabricate the micropatterned collagen scaffold. During EVPOME manufacturing, OCT was applied twice to monitor the EVPOME and evaluate its epithelial thickness.

Findings: Our moulding system resulted in successful micropattern replication on the curved collagen scaffold. OCT imaging visualised the epithelial layer and the underlying micropatterned scaffold in EVPOME, enabling to non-invasively detect specific defects not found before the histological examination. Additionally, a gradual increase in epithelial thickness was observed over time.

Conclusion: These findings demonstrate the feasibility of using a stainless-steel negative mould to create a more biomimetic micropattern on collagen scaffolds and the potential of OCT imaging for quality control in oral keratinocyte-based tissue engineering/regenerative medicine.

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1. Introduction

The development of tissue engineering and regenerative medicine has been impelled by the contribution of novel platform technologies, including scaffold-free and scaffold-based engineering [1]. However, the strategy of scaffold-based tissue engineering is much more common [2]. To this end, the donor cells are isolated and expanded to encourage proliferation under monolayer culture conditions; then, they are seeded on a three-dimensional (3D) matrix, referred to as a scaffold, for in vitro differentiation. At this point, a composite of processed cells and the specific scaffold is formed, which, after in vivo implantation, should replace or regenerate functional tissues/organs [3]. The scaffold design and composition prior to cell seeding are vital because they provide a unique microenvironment for cells, modulating their behaviour and fate. The characteristics of target tissues determine the properties of engineered scaffolds. To design and construct biologically functional scaffolds together with cellular components, a major strategy is a biomimetic approach, which includes biomimetic polymer composition (natural or synthetic) and surface properties such as chemical structures [4, 5, 6, 7]. Mechanical properties such as scaffold stiffness and topography are also considered as they create particular physical microenvironments to regulate cell fate and differentiation and enhance cell attachment and growth, especially for mesenchymal stem cells [8, 9]. On top of that, there are several emerging microfabrication technologies, including micro-milling, electrospinning, 3D printing, etc. [10, 11, 12, 13]. Therefore, selecting the appropriate biomaterial with specific scaffold modifications and proper bio-manufacturing techniques are essential to fabricating biomimetic tissue constructs.

The skin and oral mucosa primarily consist of two layers, a superficial epithelial layer and the underlying connective tissue (dermis and lamina propria), with a basement membrane in-between [14]. Their most characteristic structure is the dermo-epidermal junction (DEJ), formed by an epithelial rete ridge and its projections of the dermal papilla (connective tissue) [15, 16, 17, 18]. Therefore, this structure (dermal papilla/lamina propria) generates a unique microenvironment in the scaffolds for tissue-engineered skin and oral mucosa constructs. Despite the development of various tissue-engineered skin substitutes for 3D in vitro models and graft material, the flat interface between the scaffold and the overlying epithelial layer is not biomimetic. In contrast, previous studies showed the successful development of DEJ-like structures to investigate the epidermal keratinocyte stem cell niche [19, 20]. Lammers et al. revealed that the micro-topographic cues on the collagen membrane had a proliferative/migrative effect on epidermal keratinocytes compared with a planar collagen surface [21]. Recently, a more biomimetic, 3D undulated dermal scaffold with a topographic microstructure replicating native DEJ has been fabricated [10, 11, 12, 22]. Therefore, such scaffolds allow the construction of skin substitutes to assess cellular response under a more biomimetic environment and for clinical application in regenerative medicine.

In contrast, no studies have tried to develop biomimetic tissue-engineered oral mucosa equivalents that have great potential for translational therapy to treat oral wounds and enhance mucosal regeneration [14]. Recently, we successfully manufactured a micropatterned collagen scaffold for a biomimetic tissue-engineered oral mucosa based on histomorphology of native oral mucosa [23]. This is useful for both in vitro toxicological testing, as an alternative to animal experiments, and for clinical applications to reconstruct oral mucosa defects [13, 25]. Our procedure includes three major steps: 1) fabrication of negative moulds of a polydimethylsiloxane (PDMS) or Si substrate with micropatterns designed by micro-electromechanical systems and soft lithography; 2) production of a fish-scale type I collagen scaffold on which micropatterns were replicated; 3) manufacturing tissue-engineered oral mucosa graft on the micropatterned collagen scaffold with a DEJ-like topography [13, 24, 25].

However, multidisciplinary research is needed to provide more opportunities to improve current procedures. This type of research contributes to accelerating and translating our platform technology into the clinical setting. Therefore, this study focused on two key aspects: a) developing a system for fabricating collagen scaffolds and a regulatory framework for tissue-engineered products that thrive on advanced collaboration. A picosecond laser enables microfabrication of small cell-size level dimples on the stainless-steel mould [26]. Accordingly, this method could manufacture more biomimetic micropatterns consisting of a curved surface instead of a planar one.

Furthermore, ex vivo human cell/tissue-based products developed for use in transplantation require testing, monitoring, and inspection during in vitro culture to assure their quality. Current standard technologies usually destroy part of the 3D engineered products for monitoring and testing [27]. Thus, non-invasive tools to assess, in real-time, the in vitro viability, metabolic activity and structures are necessary for regenerative medicine [28, 29]. OCT, routinely used in ophthalmology, is an effective imaging technique that reveals tissue morphology without additional tissue fixation and labelling [30, 31]. Despite a limited penetration depth (2–3 mm), OCT has a high-scan speed and allows high-resolution cross-sectional imaging, thereby providing real-time data [32]. Therefore, OCT appears useful for monitoring and determining the 3D structure of our tissue-engineered products consisting of a topographic architecture and an overlying epithelial layer.

This study’s two main aims were to develop a picosecond laser- textured stainless-steel negative mould and to investigate the applicability and feasibility of spectral-domain OCT. The former was developed to form an undulated micropattern collagen scaffold with a curved surface that mimicked the native oral mucosa. On the contrary, the latter was used to monitor the tissue-engineered oral mucosa equivalents over time, thereby serving as a tool for quality control of a tissue-engineered oral mucosa graft.

2. Results

2.1. Topographic micropattern characterisation on stainless-steel master negative moulds

A macroscopic view of the laser-textured surface appeared black due to light reflection (Figure 1(a)). A stereomicroscopic image (Figure 1(b)) showed a well-oriented topography dominated by periodically arranged concave and convex surfaces, resembling the proposed micropattern structure. As shown in Figure 2 (a)–(c), the square and diagonal cross-sectional images obtained from the non-contact coordinate measuring machine (CMM) showed that the average depth of the different directions from the top to the bottom of the microstructure was 101.4 ± 8.8 μm and 152.4 ± 5.4 μm, respectively, and the average width between the top of the square lattice was 169.2 ± 1.5 μm. Additionally, scanning electron microscope (SEM) images showed regularly processed microstructures (Figure 3. (a)–(d)). Compared with the initially designed dimension, the picosecond laser machining, set by the parameters shown in Table 1, decreased the micropattern dimension on the master negative mould.

2.2. Characterisation of micropatterned collagen scaffolds transferred from stainless-steel negative mould

Collagen scaffolds fitting into 24-microplate wells were produced on the stainless-steel negative mould and separated from the mould (Figure 4 (a)) although the size and shape of the manufactured collagen scaffold are not limited to only 24-microplate wells and scaffolds of any size and shape can be manufactured. SEM images showed that the laser- textured microstructure on the stainless-steel negative mould was well-transferred onto the collagen scaffold and demonstrated a unique topographical feature compared with that from the Grid-Rectangular prototype of the previous study (Figure 4(b) and (c)). According to SEM, a ridge-like structure arranged in a grid pattern was formed, corresponding to the bottom grooves and
pits arranged in a square lattice in the negative mould, purple coloured in Figure 2 (a). Additionally, inside the ridge-like structure, a lacuna (crypt)-like structure was generated, corresponding to the projections in the negative mould, orange coloured in Figure 2(a). This resulted in the micropattern manufactured on the collagen scaffold appearing to be a ‘papilla’-like structure, consisting of a concave-convex surface.

2.3. OCT imaging of EVPOME as a non-invasive tool for quality control

In this study, days 8 and 11 were chosen as different epithelial stratification stages of EVPOME during manufacturing. In addition, cross-sectional images (X-Z) of the approximate ROI area were imaged to confirm location consistency over time. The representative 2D OCT cross-sectional depth images demonstrated a difference in signal intensity (Figure 5 (a)-(d)). The epithelial layer of days 8 and 11 EVPOMEs showed a strong signal. These differences in topographical characteristics between micropatterned and non-patterned scaffolds are also shown. In the micropatterned EVPOME, the area alternatively showed strong and weak signals, clearly showing the interface of the micropatterned scaffold and the overlying epithelial layer (Figure 5 (a), (c)). In the non-patterned EVPOME, the area showing a relatively homogenous weak signal indicated the collagen scaffold (Figure 5 (b), (d)). As the border between the epithelial layer and the underlying collagen scaffold was discernible, OCT images can unveil periodical ‘rete-ridge’ structures (Figure 5 (a), (c)).

Furthermore, small spots (Figure 6 (a)) and a linear gap (Figure 6 (b)) beneath the epithelial layer were occasionally found, appearing to indicate epithelial micro-detachment. Therefore, OCT imaging can detect invisible infrastructural EVPOME defects. In addition, our image capture protocol enabled us to scan multiple OCT images (day 8 and 11) for an almost identical ROI area within the EVPOME without tissue damage and contamination. These findings suggested that OCT imaging can be used non-invasively for in-process monitoring during EVPOME manufacturing. Additionally, multidirectional videos of EVPOME scan images were reconstructed using Image J to facilitate in-process monitoring (Supplementary videos 1 & 2).

Supplementary video related to this article can be found at https://doi.org/10.1016/j.heliyon.2022.e11468

2.4. Qualitative and quantitative OCT analyses on EVPOME

OCT imaging allows a qualitative and quantitative evaluation. The changes in the epithelial thickness of EVPOME over time can be a robust parameter for in-process monitoring. We explored all specific parameters regarding the epithelial layer on the micropatterned scaffold, as the pixels of the entire epithelial layer within the EVPOME were extracted by deep learning (Supplementary video 3).
Through qualitative in-process monitoring, we could successfully develop a heatmap to visualise the ROI’s epithelial thickness (Figure 7 (a), (b)). Warm-coloured circles arranged in a square lattice pattern correspond to the epithelial rete ridge, identical to the micropattern trough. In contrast, the surrounding blue area indicating a thinner epithelium corresponded to the micropattern ridge. As observed by the change in colouration, the heatmap demonstrated increasing epithelial thickness of ROI from day 8–11, indicating a regular stratification of the epithelial layer.

For quantitative measurements of the entire epithelial layer within EVPOME, the average epithelial thickness and distribution on days 8 and 11 EVPOMEs were determined. As a result, by counting approximately one million pixels in the ROI, the average epithelial thickness of the ROI on days 8 and 11 EVPOME manufactured on the micropatterned scaffold was 61.9 ± 8.6 μm and 98.6 ± 17.7 μm, respectively (Figure 8 (a)), and statistically significant. The distribution of epithelial thickness is shown in the histogram (Figure 8 (b), (c)). The peak shifted toward the right from day 8–11 EVPOME, indicating a gradual increase in epithelial thickness.

2.5. Macroscopic and microscopic findings on day 11 EVPOMEs

Out of nine EVPOME samples manufactured on non-patterned scaffolds, macroscopic evaluation of EVPOME as a final product demonstrated contraction of the overlying epithelial layer (partial detachment

| Item                  | Performance |
|-----------------------|-------------|
| Wavelength            | 532 nm      |
| Average Power         | 15 W        |
| Pulse duration        | 15 ps       |
| Beam quality M2       | <1.3        |
| Repetition rate       | 1,000 kHz   |
| Focus area            | 0.2 mm      |

Table 1. Picosecond laser processing machine settings used in this study.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.heliyon.2022.e11468

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For quantitative measurements of the entire epithelial layer within EVPOME, the average epithelial thickness and distribution on days 8 and 11 EVPOMEs were determined. As a result, by counting approximately one million pixels in the ROI, the average epithelial thickness of the ROI on days 8 and 11 EVPOME manufactured on the micropatterned scaffold was 61.9 ± 8.6 μm and 98.6 ± 17.7 μm, respectively (Figure 8 (a)), and statistically significant. The distribution of epithelial thickness is shown in the histogram (Figure 8 (b), (c)). The peak shifted toward the right from day 8–11 EVPOME, indicating a gradual increase in epithelial thickness.

2.5. Macroscopic and microscopic findings on day 11 EVPOMEs

Out of nine EVPOME samples manufactured on non-patterned scaffolds, macroscopic evaluation of EVPOME as a final product demonstrated contraction of the overlying epithelial layer (partial detachment.
from the underlying scaffold) by day 11 in six EVPOMEs. This finding contrasted with paired EVPOME samples manufactured on micropatterned and non-patterned collagen scaffolds, which the epithelial layer was not contracted until day 11 (Figure 9 (a), (b)). This structural defect was consistent with the linear gap found in OCT imaging (Figure 6 (b)), which was not able to detect before the histological examination.

Microscopic findings of day 11 EVPOMEs showed a well-differentiated, parakeratinised epithelial layer on both micropatterned and non-patterned collagen scaffolds (Figure 9 (c), (d)). The micropattern structure of the scaffold was well-preserved, similar to the picosecond laser-textured stainless-steel negative mould. The histology resembled that of a native oral mucosa, resulting in the biomimetic appearance of a rete ridge consisting of a concave-convex structure (Figure 9 (c)). However, based on the scale in Figures 5 and 9, the size of EVPOME components, such as epithelial thickness and micropattern depth, was smaller in histological images than in OCT images. This is due to EVPOME specimens shrinking after formalin fixation; thus, the EVPOME’s morphology could not be directly compared between histological analysis and OCT imaging.
3. Discussion

Several clinical trials have been conducted using cell-based products derived from oral mucosa for oral tissue regenerative therapy. However, all treatments were designed for connective tissue augmentation using oral fibroblasts, resulting in the absence of oral keratinocyte-based tissue-engineered products [33]. Additionally, grafts for oral mucosal repair become challenging following their increased introduction to load because of mastication and food [34]. Hence, the quality of the tissue-engineered oral mucosa is essential for creating a biomimetic oral epithelium-lamina propria interface [1, 34]. Previously, we reported the development of a manufacturing system to fabricate prototypes of various micro-topographic structures on collagen scaffolds, similar to the lamina propria of the oral mucosa. However, we have discovered gaps that demonstrate the need to improve the current system [13, 23]. In this study, the EVPOME composite consisting of a micropatterned collagen scaffold and an overlying epithelial layer with rete ridges was manufactured using a novel moulding system, following our human clinical application protocol. Additionally, OCT was found to be useful for the non-invasive evaluation of the EVPOME [(13, 23, 35)]. This manufacturing system resulted from the multidisciplinary collaboration between micromachining engineers and mathematical scientists.

This study confirmed that a large number of collagen scaffolds were fabricated using only one laser-textured master negative mould, which is made of certified SUS316L for medical use [36]. Additionally, the stainless-steel mould provided consistent and stable micropatterned collagen scaffolds for manufacturing EVPOME, notwithstanding the 15%–25% linear shrinkage compared to the originally designed microstructure. Therefore, the stainless-steel mould can avoid PDMS or Si substrate moulds variation, allowing large-scale scaffold production because of reusability and durability. Besides manufacturing EVPOME, the micropatterned collagen scaffold can solely serve as an acellular intraoral graft to reconstruct oral mucosa defects [22]. Hence, the laser-textured stainless-steel mould may reduce the cost and the machining time for manufacturing micropatterned collagen scaffold when taken together.

The physical properties of the stainless steel are very different from those of the PDMS used in our previous report [37]. The wettability of mould materials is a significant factor when fabricating collagen scaffolds. The water contact angle of SUS316L ranges from 50° to 75°, less than that of PDMS (100°–110°), indicating hydrophilicity [38, 39, 40, 41, 42]. Additionally, after laser machining, some studies reported an increase in the water contact angle [43, 44, 45, 46], but the others reported the opposite [40]. Therefore, SUS316L’s water contact angle remains unclear.

Figure 7. Heatmap images of ROI in day 8 and day 11 EVPOMEs depicting the epithelial layer thickness formed on a micropatterned collagen scaffold. (a) A representative heatmap depicting epithelial thickness of ROI at day 8 EVPOME. (b) A representative heatmap depicting epithelial thickness of ROI at day 11 EVPOME. Warm-coloured circular areas periodically arranged with square lattice indicate the structure of the epithelial rete ridge corresponding to the micropattern trough on the scaffold. The colouration changes from day 8 to day 11 of EVPOME implies an increase in epithelial thickness over time. Epithelial thickness was indicated by a colour bar.

Figure 8. Quantitative analysis of average epithelial thickness and a representative histogram showing distribution of epithelial thickness between day 8 and day 11 EVPOMEs generated on a micropatterned collagen scaffold. (a) A dot plot showing the average epithelial thickness of ROI on days 8 and 11 EVPOMEs (N = 9). The red line indicates the average of a total of nine specimens. There was a significant difference in the average epithelial thickness between day 8 and day 11 EVPOMEs, demonstrating the increase in epithelial thickness over time. (b) A representative histogram showing epithelial thickness of day 8 EVPOME. (c) A representative histogram showing epithelial thickness of day 11 EVPOME. Compared with day 8 EVPOME, the peak of the histogram shifts to the right, indicating a gradual increase in epithelial thickness. Those data were non-invasively acquired from OCT imaging.
Moreover, there are no standard data on the contact angle of the collagen solution. Our pilot data of contact angle with 1.1% collagen solution showed that both the microstructured and the non-structured surfaces of SUS316L were hydrophilic, and this hydrophilicity increased over time. To our knowledge, this is the first report to apply picosecond laser microstructuring to SUS316L; the wettability of the microstructured SUS316L surface to the collagen solution appeared to facilitate the use of our technology.

Poor micropattern transfer and replication onto the collagen scaffold was a major technical concern initially when separating the collagen scaffold from the mould since the stainless-steel plate is unbendable, unlike pliable PDMS. Despite no technical issues, this might result from the rheological properties of collagen, such as viscoelasticity and stiffness, similar to the previous report (data not shown). Thus, the affinity between the physical and mechanical properties of the laser-textured stainless-steel surface (wettability and roughness) and collagen could facilitate the separation. Nevertheless, further research is required to examine the dynamics of the interface between the collagen scaffold and the SUS316L depending on the microstructure topography of the stainless steel and its complement micropattern of the collagen scaffold.

Here we developed a 3D papillary structure on a collagen matrix mimicking the lamina propria and having a curved surface for EVPOME manufacturing. However, future studies are required to develop new biomimetic micropatterns with an irregular shape and size, as the micropattern fabricated here follows a regular pattern, in contrast to in vivo tissue showing topographic variations of the dermal papilla and lamina propria ([17, 18, 47]). Nonetheless, our technology allows investigating the interaction between the undulation of the connective tissue and the fate of basal layer cells in the overlying epithelium, especially keratinocyte stem cell differentiation [9, 12, 48, 49, 50]. More importantly, the micropatterned scaffold fabricated in this study lacks a basement membrane at the interface of the overlying epithelium [12, 17]. Biochemical research strategy is required to incorporate or bond basement membrane components, such as laminin and perlecan, to collagen fibrils at the molecular base.

Furthermore, concerning the regulatory framework in regenerative medicine, a standardising protocol for manufacturing products and ensuring quality control is mandatory to boost clinical translation. This framework involves good manufacturing practices [51]. Additionally, for quality control, non-invasive methods are essential to evaluate tissue-engineered soft tissues in 3D culture setups. Currently, this process does not require any special sample preparation, such as labelling before use and preserving the sample during manufacturing products. Currently, a few tools enable in-process monitoring of 3D constructs and non-invasive evaluation of the end product ([26, 52, 53, 54, 55]). Previously, we reported using Raman spectroscopy for in vitro monitoring of the viability of tissue-engineered constructs. However, the lack of standardisation limits its translation into the clinic [56, 57]. In this study, OCT imaging was a valuable tool for EVPOME quality control. It enabled the visualisation of the epithelial layer morphology and the underlying scaffold in the EVPOME with a high spatial and temporal resolution, consistent with findings from the previous study [58]. In addition to visualising the EVPOME infrastructure, small spots and a linear gap between the epithelial layer and underlying collagen scaffold may result in EVPOME fabrication failure caused by malnutrition or epithelial detachment. This indicated that OCT could image possible invisible defects that cannot be detected by routine macroscopic observation. Therefore, considering that OCT imaging can be a promising tool, further
translation of non-invasive methodologies for regenerative medicine is essential to bring those effects closer to their clinical application.

Furthermore, although our OCT scanning protocol allows preservation of the EVPOME sample during the analysis, it might subject the sample to some stress, which could invalidate the final quality [51]. Technically, the EVPOME was placed upside down for scanning to obtain sufficient resolution of the epithelial layer due to the limited light penetration depth. For multiple analyses with maintaining EVPOME integrity, special care is currently needed when handling the sample, which could be the one to be improved.

Furthermore, quantitative evaluation of the constructs is highly recommended for adequate clinical translation. Therefore, this will enable the effective utilisation of OCT imaging for quality control of tissue-engineered epithelial tissues. Epithelial thickness is one of the specific parameters to be measured in the study [19, 59]. Fewer studies on tissue engineering in relation to bladder urothelium differentiation and a 3D gut model measured the epithelial thickness using OCT imaging [60, 61]. Additionally, an investigative study on the skin equivalent demonstrated the OCT measurement of the specific layer thickness [62]. However, these studies only measured a single layer of the epithelium.

In contrast, the measurement of the thickness and its distribution on days 8 and 11 EVPOME was complicated because the epithelial layer formed on the micropatterned scaffold had rete ridges. Moreover, this required discriminating the epithelial layer having rete ridges from the underlying undulated collagen scaffold on OCT images. However, this study achieved this by applying deep learning to OCT images to recognise the DEJ [63]. Meanwhile, this is the first report to determine the inter-face, notwithstanding the difficulty of visualising DEJ in the OCT imaging [27]. When combined with deep learning, the utility and feasibility of OCT imaging contribute to oral keratinocyte-based regenerative medicine and thus accelerate clinical translation. However, further studies are needed for optimal epithelial regeneration to find alternative key biologic parameters and to apply different imaging techniques for EVPOME quality control [64].

Despite of morphological similarity of the day 11 EVPOME formed on micropatterned scaffold between the microscopic and OCT images obtained from the same sample, the thickness of the two layers of the epithelial layer and main collagen scaffold in the microscopic images was smaller than in OCT images. Since the live OCT image is highly likely to display the actual EVPOME size, the histologic examinations of EVPOME, including our previous studies, where we concluded the micropattern was deformed, could have resulted from misinterpretation [13, 23]. This is consistent with the previous reports comparing the utility of OCT images with conventional histologic examination on skin equivalents [58, 65]. Shrinkage of paraffin-embedded specimens caused by fixation and dehydration is more or less unavoidable. Accordingly, there is a likely contraction and deformation of the micropattern histologic appearance and the collagen scaffold at day 11 EVPOME. Therefore, this study elucidated the discrepancies between histologic examination and OCT imaging of day 11 EVPOME. Closer attention should be paid to the differences caused by these two images to evaluate the morphology of the two layers. According to a previous study on skin equivalents demonstrating a higher correlation of epidermal layer thickness between the microscopic and OCT images [66], cryosection may solve the discrepancy while those skin equivalents, including the epidermal equivalent, do not have micropatterning in the scaffold [62].

Since a few decades, OCT has been clinically used to examine oral soft tissues in vivo, especially in oral mucosa lesions [67, 68, 69]. The pathological changes in vivo examined using swept-source frequency-domain OCT, different technical approach from this study, were able to identify different tissue pathologies [69]. Owing to its high-resolution imaging of mucosal surface, OCT is useable for wound healing monitoring after EVPOME intraoral grafting using a specific probe.

4. Conclusion

We successfully developed a novel technique for fabricating micropatterned collagen scaffolds, similar to the lamina propria of the oral mucosa. A picosecond laser-textured stainless-steel master negative mould was fabricated, and its undulated microstructure was replicated onto a fish-scale collagen scaffold. The scaffold micropattern, consisting of a curved surface, allowed the manufacturing of an EVPOME composite with rete-ridge structures mimicking the in vivo oral mucosa.

We also demonstrated the applicability and feasibility of OCT imaging as a quality control tool during EVPOME manufacturing, allowing in-process monitoring for non-invasive and quantitative evaluation of living products over time.

These two pioneering initiatives for regenerative medicine, biomimetic micropatterned collagen scaffolds and quality control systems, could improve oral keratinocyte-based tissue-engineered products.

5. Material and methods

Ethical approval

The tissue procurement of oral mucosa samples and tissue use was authorised by the Internal Review Board of the Niigata University Hospital (Approval # 2015–5018). All procedures were committed to relevant guidelines and regulations, such as Ethical Guidelines for Medical and Biological Research Involving Human Subjects.

5.1. Micropattern design and fabrication on stainless-steel master negative moulds using a picosecond laser processing machine

To produce a biomimicking micropatterning of the lamina propria of oral mucosa on fish-scale collagen scaffolds, we designed an undulated pattern with a periodically-arranged square lattice comprising a curved surface of 134-μm depth and 200-μm width. This design demonstrates a more biomimetic topography of tissue-engineered oral mucosa due to long vertical micropatterns [13, 23]. After setting the laser output power at 90 mW, the wavelength was 532 nm, the pulse <15 ps and the frequency 20 kHz; the designed micropattern was machined on a stainless-steel plate (SUS316L: Steel Use Stainless 316 Low carbon) (Komatsuseiki Kosakusho Co., Ltd., Suwa, Nagano, Japan), 50 mm × 50 mm in size and 0.4 mm thick, using a picosecond laser processing machine (PANASONIC AP-3220, PANASONIC, Osaka, Japan). Machining was performed only on the central area of the plate, utilised as a master negative mould in this study. The machining area was 16.5 mm × 16.5 mm square in which one well size of 24 microplates (Corning, New York, NY, USA) is inscribed.

The shape and dimensions of the micropattern fabricated by laser machining on the stainless steel were photographed by stereoscopic microscopy (SMZ745, NIKON, Tokyo, Japan) and measured by a non-contact coordinate measuring machine (CMM) (INFINITE FOCUS G5:...
5.2. Preparation of micropatterned fish-scale collagen scaffolds

The size of a circular fish-scale collagen scaffold manufactured in this study was 16 mm in diameter and 1 mm in thickness. The scaffold used in this study consisted of 1.1% type I tilapia scales atelocollagen matrix with 1% 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDC) chemical crosslinking for better handling than the previously reported one [13]. Micropatterned and non-patterned (flat, control) collagen scaffolds were prepared [13] by placing a silicone rubber sheet (1.0 mm in thickness) (Asone Corporation, Osaka, Japan) with a punched-out round hole (16 mm in diameter) on the stainless-steel plate as mould. Cell campus (100% freeze-dried collagen; FD-08G, Taki Chemical Co., Ltd., Hyogo, Japan) was dissolved with HCl. The collagen solution was mixed with Dulbecco's phosphate-buffered saline (D-PBS, KAC Co., Ltd, Kyoto, Japan) at 4 °C, poured into the round hole of silicone rubber space as a master mould with laser-textured stainless steel, and transferred into an incubator (25 °C) to induce fibrogenesis. Subsequently, the scaffolds were separated from the stainless-steel master mould, immersed in a 1% EDC solution for chemical crosslinking as previously reported, and washed in 50% ethanol followed by D-PBS by rotational stirring at room temperature for 24 h. Then, they were all γ-irradiated for sterilisation. Control scaffolds were also prepared on a flat stainless-steel plate. SEM showed the micropatterned scaffolds, and the storage and loss modulus were determined using a rheometer (HAAKE MARS III, Thermo Fisher Scientific Inc., Germany) as described previously [13, 23].

5.3. Procurement of oral tissue samples

Patients who underwent the third molar tooth removal and/or minor dental/veal surgery at the oral and maxillofacial surgery out-patient clinic in Niigata University Hospital received sufficient information regarding this study; all participating individuals signed an informed consent form. An oral mucosal specimen approximately 5 mm² in size was obtained from consenting individuals.

5.4. Cell culture of primary oral keratinocytes

Serial primary oral keratinocyte culture was performed as reported previously [13, 23]. Briefly, the oral mucosa sample was mechanically cleaned and soaked in 0.025% trypsin ethylenediaminetetraacetic acid solution (Thermo Fisher Scientific, Waltham, MA, USA) containing 1.5% Antibiotic-Antimycotic (Thermo Fisher Scientific) for 16 h at room temperature. Then, we transferred the specimen into a 0.0125% defined trypsin inhibitor (Thermo Fisher Scientific) solution, and the epithelial layer was scraped off from the underlying connective tissue using a scalpel. Dissociated oral keratinocytes were resuspended in a ‘complete medium’, Epilife® supplemented with Epilife® Defined Growth Supplements (Thermo Fisher Scientific) containing Gentamicin and Amphotericin B (Thermo Fisher Scientific) and plated at a density of 4.0–5.0 × 10⁶ cells/cm². They were fed with a complete Epilife® culture medium every other day. When reaching 70%–80% confluence, they were re-plated at a density of 0.7–1.0 × 10⁵ cells/cm² and serially subcultured. The medium was refreshed every other day. This experiment used oral keratinocytes from passages 2 to 4 (p2–p4).

5.5. Manufacturing an ex vivo produced oral mucosa equivalent (EVPOME)

To manufacture the EVPOMES, oral keratinocytes at p2 to p4 were seeded onto a micropatterned and a flat surface (as a control ex vivo produced oral mucosa equivalent (EVPOME)) collagen scaffold at a density of 1.5 × 10⁵ cells/cm². Briefly, the collagen scaffolds were pre-soaked with type IV collagen (Sigma-Aldrich, St. Louis, MO, USA) and D-PBS (Wako Chemical, Osaka, Japan) overnight at four °C in a 24 well-plate according to our human clinical application protocol [25]. After seeding oral keratinocytes onto the scaffolds, cell-scaffold composites were cultured in a submerged condition. They were fed daily with a complete medium supplemented with 12 mM Ca²⁺ for 4 days, raised to an air–liquid interface, and cultured with the same culture medium for another 7 days by refreshing the medium every other day. Nine pairs of EVPOMEs, formed on micropatterned and non-patterned control collagen scaffolds, were manufactured; during manufacturing, the identical EVPOME samples were examined using OCT imaging on days 8 and 11.

5.6. Scanning protocol of tissue-engineered oral mucosa samples (day 8 and day 11 EVPOME) and optical coherence tomography (OCT) imaging analysis

A spectral-domain OCT (SD-OCT) system, Cell®/Mager Estier (SCREEN Holdings, Kyoto, Japan), was used to observe the cross-section of 3D-tissue specimens during manufacturing EVPOME over time. The system is equipped with a light source from a super luminescent diode (SLD) with a centre wavelength of approximately 900 nm and output power of 10 mW. The SLD output is coupled into a single-mode optical fibre, split into the EVPOME and reference arms at the fibre coupler. The reflections from the two components are combined at the coupler and identified by the spectrometer. OCT images of days 8 and 11 EVPOME were captured every 1 μm along the Z-axis from the epithelial layer to the underlying micropatterned collagen scaffolds using a high-magnification lens; the actual focus positions were calibrated using the nominal focus positions and the difference between refractive indices. OCT image data sets in the axial and transversal planes at a resolution of 1 μm were prepared. The X–Y scan area was 1000 μm × 1000 μm. The Z-axis scan depth was approximately 200–300 μm.

On OCT imaging, the circular EVPOME sample was transferred into a 60 mm culture dish with a 2-mm grid (Corning) using forceps and placed in the centre of the dish with the epithelial surface down. A total of 1 mL of culture medium was added to the dish to maintain the moisture during scanning. Additionally, to ensure the OCT imaging position for day 11, four locations on day 8 EVPOME crossing the dish reticle were tattooed with Indian ink. After the dish was set in the OCT microscope, we chose a region of interest (ROI) (1 mm × 1 mm) in one grid (2 × 2 mm) at the central area of day 8 of EVPOME. After completing OCT image capture, the sample was cultured again for further manufacturing. On the second OCT imaging for the same EVPOME on day 11, we prepared the sample as described previously. This allowed us to determine the approximate ROI area of the day 8 EVPOME and use the same OCT scan settings, ensuring OCT imaging repeatability.

All images were scanned at 1 μm/pixel resolution. Cross-sectional EVPOME images, including the epithelial layer and the underlying micropatterned scaffold, were binarised using semantic segmentation, for which we used a convolutional neural network, one of the major deep learning algorithms [48,63]. Image processing by overlaying the binary image onto the original one enabled us to extract only the epithelial layer, exclude the scaffold area, and evaluate the morphological features of the epithelial layer within EVPOMEs using ImageJ (National Institutes of Health, Bethesda, MD, USA). After constructing a 3D image including only the extracted epithelial layer, converting X-Z to X-Y slice images created a heatmap of the epithelial layer that can overlook and visualise the ROI’s ‘epithelial thickness’. Quantitative analyses based on OCT images were conducted to determine changes in the spatiotemporal characteristics of the epithelial layers within the 3D-ROI of the EVPOME during manufacturing, specifically ‘average epithelial thickness’ and ‘distribution change of epithelial thickness’. Analyses were performed based on the grey levels of OCT X-Z images.
slice images within the entire 3D-ROIs. Because the 8-bit image has 256 grey levels, referred to as a Bin, the number of pixels from Bin 1 to 256 (1 μm/pixel) within 3D-ROIs was counted (approximately one million pixels).

5.7. Histological examination of EVPOMEs

After obtaining OCT images of day 11 EVPOMEs, the ROI was tattooed with Indian ink on the back surface of the EVPOME by looking at the screenshot acquired on days 8 and 11; the day 11 EVPOMEs were fixed with 4% paraformaldehyde in 100 mM D-PBS. Three incisions were applied onto the specimen and were cut into four pieces. The ROI area tattooed was included in the middle incision line to ensure the ROI area for identification of the OCT image to the histologic specimen of the day 11 EVPOME. After dehydration, specimens were embedded in paraffin. These paraffin-embedded specimens were cut into 5-μm sections, deparaffinized, rehydrated, and stained with haematoxylin and eosin for histological analysis.

5.8. Statistical analysis

A paired t-test was used to analyse the differences in average epithelial thickness between the EVPOMEs cultured for 8 and 11 days using Excel software (n = 9). A p-value <0.05 was considered to indicate significant differences.

Declarations

Author contribution statement

Orakarn Suebsamarn, Yoshifumi Kamimura, Kenji Iizumi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Yoshihiro Kodama, Ryouuke Mizuno: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ayako Suzuki: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Yasushi Osawa: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Takafumi Komatsu: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Kazuma Kishimoto: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Jun Mizuno: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Taisuke Sato, Kenta Haga, Ryota Kobayashi, Eriko Naito, Haruki Hayasaki: Performed the experiments; Analyzed and interpreted the data.

Masakazu Kida: Conceived and designed the experiments; Analyzed and interpreted the data.

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Data availability statement

All experimental data obtained in this study are available upon direct and reasonable request to the corresponding author. All the data supporting the findings of this study are included within the article and supplementary materials.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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