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# Development, optimization and characterization of a full-thickness tissue engineered human oral mucosal model for biological assessment of dental biomaterials

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**Abstract** Restorative dental materials and oral health care products come into direct contact with oral mucosa and can cause adverse reactions. In order to obtain an accurate risk assessment, the in vitro test model must reflect the clinical situation as closely as possible. The aim of this study was to develop and optimize a three-dimensional full-thickness engineered human oral mucosal model, which can be used for biological assessment of dental materials. In this study human oral fibroblasts and keratinocytes were isolated from patients and seeded onto a number of collagen-based and synthetic scaffolds using a variety of cell seeding techniques and grown at the air/liquid interface to construct human oral mucosa equivalents. Suitability of 10 different scaffolds for engineering human oral mucosa was evaluated in terms of biocompatibility, biostability, porosity, and the ability to mimic normal human oral mucosa morphology. Finally an optimized full-thickness engineered human oral mucosa was developed and characterized using transmission electron microscopy and immunostaining. The oral mucosa reconstruct resembled native human oral mucosa and it has the potential to be used as an accurate and reproducible test model in mucotoxicity and biocompatibility evaluation of dental materials.

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## 1 Introduction

Commonly used cell culture systems for the assessment of the biocompatibility of dental materials are primarily based on monolayer cell culture models that do not mimic the anatomy of the oral epithelium and connective tissue. Full thickness skin models have been developed to evaluate cutaneous toxicity and efficacy of cosmetic products [1, 2]. However, three-dimensional oral mucosal models available on the market (Epioral/Epigingival [MatTek, Ashland, MA] and SkinEthic oral epithelium [SkinEthic laboratories, Nice, France]) consist only of partial thickness oral epithelium without underlying connective tissue components. Both the connective tissue and epithelium, and their interactions, are essential factors that must be considered in the biological evaluation of biomaterials. Underlying fibroblasts play an important role in epithelial differentiation [3], keratinocyte adhesion and formation of the complex dermal-epithelial junction by synthesizing extracellular matrix (ECM) [4]. Furthermore, the epithelial phenotype and profile of cytokeratin expression is influenced by the nature and origin of the underlying mesenchymal substrate and fibroblasts [5–7].

Tissue-engineered oral mucosal equivalents have been developed for clinical applications and also for in vitro studies of biocompatibility, mucosal irritation, disease, and other basic oral biological phenomena [8]. Several studies have reported successful assembly of full-thickness engineered human oral mucosa by culturing oral keratinocytes, with or without fibroblasts on collagen [9–11] or processed cadaver skin scaffolds such as de-epidermized dermis (DED) [12, 13] and AlloDerm [14]. There are several limitations associated with the use of processed cadaver skin as a scaffold for an oral mucosal model. DED is obtained from different people and different sites of the

body that might have different thickness and characteristics. This may compromise the accuracy of the test model, and there are issues with regard to fibroblast infiltration and migration across the membrane in vitro. There are also some ethical issues regarding the use of human skin. AlloDerm shares most of the characteristics of DED and it is specifically designed for surgical transplantation purposes. As a result, it is expensive and an in vitro test model based on this scaffold would not be cost-effective. Furthermore, both systems use a skin derived connective tissue that, given the influence of the origin of the connective tissue on epithelial morphology [5], could adversely affect the development of a full thickness model of the oral mucosa. On the other hand, collagen-based scaffolds would be suitable for this purpose. However, there is no comprehensive study comparing different types of collagen-based and synthetic scaffolds used for engineering human oral mucosa and no optimized, reproducible full-thickness oral mucosal test model has been developed for in vitro biological assessment of dental materials. The aim of this study was to develop and optimize a three-dimensional tissue engineered human oral mucosal model, which could be used for biological assessment of dental materials. This study was approved by the South Sheffield Research Ethics Committee.

## 2 Materials and methods

### 2.1 Keratinocyte culture

Biopsies (5 × 5 mm) from keratinized palatal mucosa were obtained with written informed consent from patients having surgical exposure of unerupted canine teeth. First, keratinocytes were enzymatically isolated from the biopsies by incubating the tissue in a solution of 1% trypsin 1:250 (Difco, UK) in phosphate-buffered saline (PBS) (SIGMA, UK) for 1h at 4 °C followed by 2 h at 37 °C. The epithelial surface was then scraped with a scalpel and a direct explant technique used to obtain the maximum number of keratinocytes from the remaining epithelium. In this technique tiny pieces of epithelium were individually placed in a T-25 tissue culture flask and allowed to attach for 15 min. The keratinocytes were then cultured in Green's medium in a humidified atmosphere of 5% $CO_2$/95% air at 37 °C. Green's medium consisted of Dulbecco's Modified Eagle Medium (DMEM) (SIGMA, UK) and Ham's F-12 medium (SIGMA, UK) in a 3:1 ratio, supplemented with 10% fetal calf serum (FCS) (Biowest Ltd., UK), 10 ng/mL epidermal growth factor (EGF) (SIGMA, UK), 0.4 μg/mL hydrocortisone (SIGMA, UK), $10^{-4}$ mol/L adenine (SIGMA, UK), 5 μg/mL insulin (SIGMA, UK), 5 μg/mL transferring (SIGMA, UK), $2 \times 10^{-7}$ mol/L triiodothyronine (SIGMA, UK), $2 \times 10^{-3}$ mol/L glutamine (SIGMA, UK), 2.5 μg/mL fungizone (SIGMA, UK), 50 U/mL penicillin (SIGMA, UK) and 50 U/mL streptomycin (SIGMA, UK).

The cells were fed two to three times a week until confluent. Keratinocytes were used after the second passage to produce the oral mucosal model.

In addition to normal oral keratinocytes, the suitability of the TR146 keratinocyte cell line was also examined for producing an engineering oral mucosa on an optimized scaffold. The immortal human TR146 cell line was derived from a neck lymph node metastasis originating from a carcinoma of the buccal oral mucosa [15]. These cells were kindly provided by Cancer Research UK.

### 2.2 Fibroblast culture

Fibroblasts were isolated from the connective tissue layer of the biopsies by incubating in 0.05% (w/v) collagenase type I solution in DMEM containing 10% FCS at 37 °C overnight. Fibroblasts were cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, 625 ng/mL fungizone, 50 U/mL penicillin and 50 U/mL streptomycin. Fibroblasts were used after the third passage in order to enhance the reproducibility of the oral mucosal model. Another reason for using the fibroblasts at an early passage number was that the extracellular matrix production by fibroblasts decreases as the passage number increases [16, 17].

### 2.3 Scaffolds

A number of natural and synthetic scaffolds were investigated for their suitability in the construction of the human oral mucosal model in 10 separate experiments. The commercially available scaffolds included:

a. Collagen type I membrane (Collatape, Calcitek, USA).
b. Collagen type I cross-linked with 0.25% glutaraldehyde (Agar Scientific Ltd., Essex, UK) for 24 h.
c. Collagen types I and III bilayer membrane (Bio-Gide, Geistlich, Switzerland).
d. Collagen-elastin membrane (Permacol, Tissue Science Laboratories plc, UK).
e. Poly (ethylene terephthalate) (PET) membrane (Greiner bio-one, Germany), pore size 3.0 μm.
f. Polycarbonate (PC) membrane (Costar, USA), pore size 3.0 μm.

In addition a number of experimental scaffolds were constructed as described below:

g. Electro-spun poly L-lactic acid (PLLA): A 8% (w/w) solution of PLLA in dichloromethane (DCM) was prepared and spun (voltage: 15 kV, working distance: 15 cm). Fibers were collected on an aluminum foil ($18 \times 16$ cm$^2$) using an earth rotator. Jet alignment was assisted by an aluminum focusing ring. Both laminated and non-laminated scaffolds were examined. Lamination of this scaffold was performed by adding 50 μL of Matrigel (BD Biosciences, USA) onto the surface and incubating at 37 °C for 30 min to induce gel formation before keratinocyte seeding.
h. Electro-spun polystyrene: A 30% (w/w) solution of polystyrene (PS) in tetrahydrofuran (THF) was prepared and spun (voltage 25 kV, working distance 15 cm) as described above. Instead of laminating the scaffold with Matrigel, fibroblasts were mixed with Matrigel and incorporated into the scaffold on ice and then incubated at 37 °C to gel. Since polystyrene is soluble in xylene, which is used in H&E staining, the sections of this oral mucosal model were not treated with xylene and instead were mounted using an aqueous mounting medium (Immu-Mount, Shandon, USA) when being processed for histological assessment.
i. Agarose gel: 4% agarose (SIGMA, UK) was made in PBS and sterilized by autoclaving. The gel was melted, cooled to 37 °C and then mixed rapidly (50:50) with a fibroblast cell suspension of $6 \times 10^6$ cells/mL medium and immediately dispensed into 24 well cell culture inserts (200 μL).
j. Collagen-glycosaminoglycan (GAG)-Chitosan (CGC) porous scaffold: collagen type I from bovine skin, chitosan (95% deacetylated), chondroitin-6-sulfate, and hyaluronic acid (high molecular weight) were purchased from SIGMA, UK. Collagen and chitosan were dissolved in 0.1 M acetic acid to prepare 0.5% (w/v) solutions. Other substances were dissolved in distilled water to prepare 0.5% concentrations. These solutions were mixed with the following ratio and homogenized: collagen/chitosan/GAG/HA: 100/12/5/1. The mixture was then neutralized by sodium hydroxide 0.5 M solution and then injected into 48 well plates. After de-aeration under vacuum, it was incubated at 37 °C for 6 h. Then it was frozen at −80 °C and then freeze-dried for 24 h to obtain a porous scaffold. The scaffolds were sterilized by $\gamma$-irradiation and cross-linked with filter sterilized 0.25% glutaraldehyde solution for 24 h. Then they were rinsed with distilled water five times and immersed in the PBS for 24 h and then immersed in the DMEM before seeding with fibroblasts. The scaffold was laminated before keratinocyte seeding using Matrigel (see g above).

## 2.4 Oral mucosa model

A sheet of the scaffold ($15 \times 15$ mm) was placed into a 6-well plate and a 1 cm diameter stainless steel ring was placed at the center of the scaffold. Then 1 mL of the fibroblast cell suspension ($5 \times 10^5$ cells/mL) was added to the inside of the ring. At the same time 3 mL of DMEM was added to the outside of the ring to maintain pressure balance inside and outside the ring (Fig. 1a and b). After incubation at 37 °C for 24 h, the ring was removed and the medium was replaced. Cells were further incubated for 1 week. The scaffold was inverted and placed into a 6-well tissue culture insert with the fibroblasts towards the base of well. The insert was placed into a 6-well plate, a stainless steel ring was placed in the center of the scaffold and 1 mL keratinocyte cell suspension ($1 \times 10^6$ cells/mL) was added to the inside of the ring (Fig. 1c). After 24 h the ring was removed and the medium was replaced. The composite was

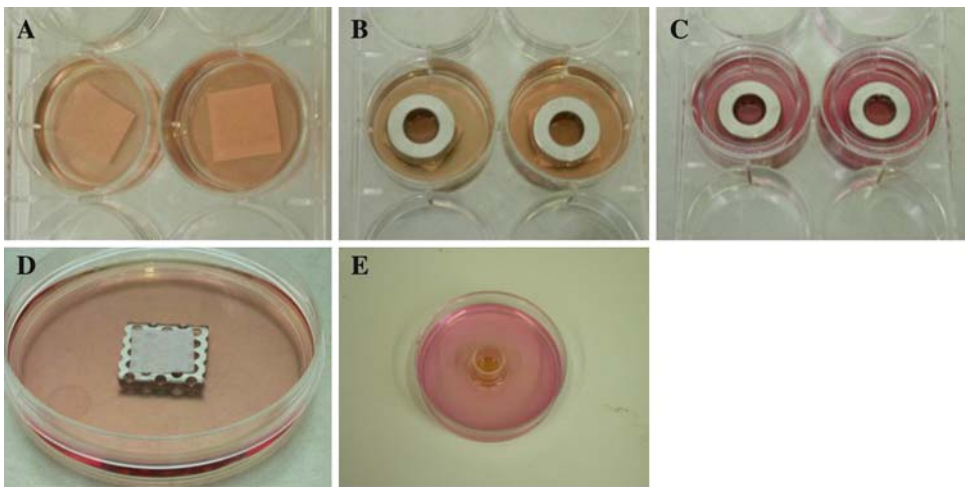


**Fig. 1** Different stages of cell seeding and culture; (**a**) scaffolds in the medium, (**b**) fibroblasts seeded onto scaffolds using steel rings to confine the cells to the scaffold surface; (**c**) the scaffolds are inverted and placed on cell culture inserts, with fibroblasts facing down, and keratinocytes are seeded onto the upper surface using steel rings to confine the cells. (**d**) The mucosal model cultured at the air–liquid interface. (**e**) Mucosal models cultured at the air–liquid interface using a CGC scaffold in a cell culture insert

**Table 1** Summary of the scaffolds tested, cell seeding techniques, and corresponding results

| Scaffold | Cell seeding technique | Biocompatibility | Biostability | Porosity/thickness | Epithelial morphology |
|---|---|---|---|---|---|
| Collagen type I | 2 sided<br>2 step 1 sided<br>1 step 1 sided | Good | Less than 2 weeks | Poor fibroblast infiltration into scaffold | Multi-layer on compact areas and epithelial islands on porous areas |
| Cross-linked collagen type I | 2 sided | Good | Good | Same as above | Same as above |
| Bilayer collagen types I and III | 2 sided | Good | Less than 3 weeks | Too thick | Keratinocyte multi-layer |
| Collagen-elastin | 2 sided | Good | Good | Very poor porosity | Thin epithelial layer |
| PET and PC membranes | 2 sided | Good | Good | No 3D matrix for fibroblast support | 2-3 layers of keratinocytes |
| Electro-spun PLLA | 2 step 1 sided | Good | Good | Good | Multi-layer in laminated scaffold, island formation in non-laminated scaffold |
| Electro-spun PS | 2 step 1 sided | Good | Good | Larger pore size than PLLA | Thin epithelial layer |
| Agarose gel | 2 step 1 sided | Poor cell adhesion | N/A | N/A | No epithelium present |
| CGC | 2 step 1 sided | Good | Good | Good | Multi-layer stratified epithelium |

cultured while submerged for 3 days and was then placed on a stainless steel grid in a petri dish and maintained at the air/liquid interface for 10 days (Fig. 1d). For the following scaffolds: PET (e), PC membrane (f), electrospun PLLA (g), electrospun PS (h), agarose gel (i) and CGC (j) scaffolds, cell culture inserts (24 well) were used instead of stainless steel rings and grids to maintain growth of the oral mucosal model at the air–liquid interface (Fig. 1e).

With regard to the collagen I scaffold (scaffold a), which had a porous and a compact side, three cell seeding techniques were studied: double-sided cell seeding (fibroblasts on the porous side and keratinocytes on the compact side), two-step single-sided cell seeding (first fibroblasts on the porous side and then keratinocytes on the same side), and one-step single-sided seeding (fibroblast and keratinocyte co-culture on the porous side). A two-step single-sided cell seeding technique was employed with the electro-spun PLLA (g), PS (h) and CGC (j) scaffolds (Table 1).

Each model was cultured for 10 days and fed every 2–3 days by removing the spent medium and replacing it with fresh medium. Different samples of each engineered oral mucosal model were then processed for routine histological evaluation, electron microscopy and immunofluorescence studies.

## 2.5 Histology

Engineered oral mucosal models were frozen in liquid nitrogen, sectioned (8 μm), fixed with 4% paraformaldehyde solution in PBS and stained with haematoxylin and eosin (H&E). Histological sections of the engineered oral mucosal models were examined under a light microscope by multiple observers and random pictures of the sections were obtained for each model. Scaffolds with promising histological results were chosen for transmission electron microscopy examination and immunofluorescence study.

## 2.6 Transmission electron microscopy

Tiny pieces of engineered oral mucosa (size 2 mm) were cut, fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h at 4 °C, and postfixed in 1% osmium tetroxide. Tissue was then dehydrated with ethanol and embedded in epoxy resin (Agar 100). The sections were examined using a Philips CM10 transmission electron microscope.

## 2.7 Immunofluorescence study

Frozen sections (8 μm) were fixed with acetone/methanol (1:1) blocked with 10% normal donkey serum in PBS and incubated with one of the following primary monoclonal antibodies: mouse anti-human cytokeratin 5 (1:150), mouse anti-human cytokeratin 10 (1:50), mouse anti-human cytokeratin 13 (1:300), and mouse anti-human cytokeratin 19 (1:100). They were then washed two times with PBS

and incubated with fluorescein (FITC)-conjugated donkey anti-mouse secondary antibody (1:20). All of the antibodies were purchased from Vector laboratories, Burlingame, CA.

Normal gingival mucosa was used as positive control and the primary antibody was omitted in negative controls.

## 3 Results

Results of the analysis of the engineered oral mucosal models based on different scaffolds are summarized in Table 1.

### 3.1 Histology

Results of the histology for each of the commercially supplied scaffolds were as follows:

#### *3.1.1 Collagen type I scaffold*

Oral keratinocytes formed stratified squamous epithelium with 9–12 cell layers. Multi-layers of keratinocytes were formed on the dense surface areas of the collagen matrix but epithelial cells infiltrated into the scaffold in porous areas and formed epithelial islands (Fig. 2a). Although epithelial island formation was observed frequently, few fibroblasts were observed inside the pores of the scaffold regardless of the seeding technique used. However cultured, this engineered mucosa did not survive more than 2 weeks due to biodegradation of the scaffold.

#### *3.1.2 Cross-linked collagen*

Bio-stability of the collagen scaffold was improved by chemical cross-linking and the engineered mucosa survived for more than 3 weeks. However, the histology was similar to that seen with the collagen type I scaffold (Fig. 2b).

#### *3.1.3 Collagen types I and III bilayer membrane*

Keratinocytes grew on the compact layer and some fibroblasts grew into the porous layer of the membrane. Since the scaffold was too thick, fibroblasts were unable to pass through both layers of the membrane (Fig. 2c). The scaffold curled up a few days after seeding with fibroblasts and it was biodegraded within 3 weeks of initial cell seeding.

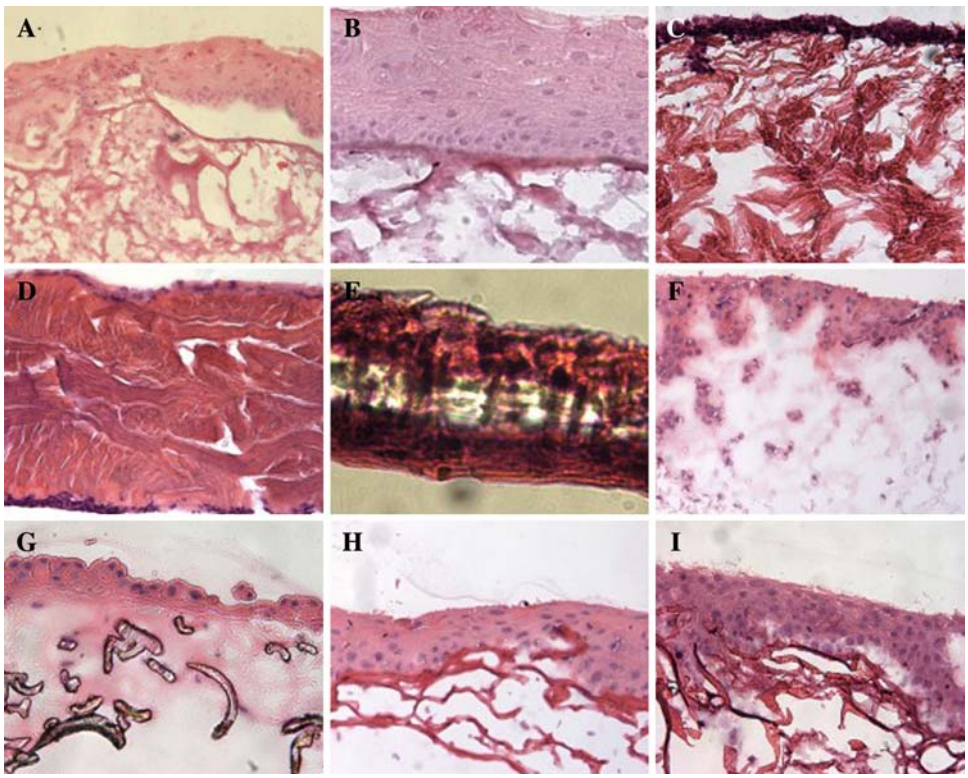


**Fig. 2** Histology of H&E stained sections of engineered oral mucosa on different scaffolds; (**a**) on collagen type I scaffold showing keratinocyte multi-layers on dense areas of the scaffold matrix and cell infiltration and island formation on porous areas of the scaffold; (**b**) cross-linked collagen type I; (**c**) collagen types I and III bi-layer membrane; (**d**) collagen-elastin membrane; (**e**) PET membrane; (**f**) electro-spun PLLA; (**g**) electro-spun polystyrene; (**h**) collagen-GAG-chitosan matrix with normal oral keratinocytes; and (**i**), collagen-GAG-chitosan matrix with the TR146 cell line

#### 3.1.4 Collagen-elastin membrane

Only a thin layer of epithelial cells grew on the surface of the scaffold and fibroblasts were unable to penetrate into the scaffold due to its lack of porosity (Fig. 2d). The scaffold did not biodegrade during this period.

#### 3.1.5 PET membrane

Keratinocytes and fibroblasts grew on both sides of this membrane and were able to interact with each other through the pores of the membrane (Fig. 2e).

#### 3.1.6 Polycarbonate membrane

The result was the same as PET membrane.

Results of the histology for experimental scaffolds are described below:

#### 3.1.7 Electro-spun PLLA

This scaffold showed good biocompatibility and bio-stability. In the non-laminated version of the scaffold, epithelial cells infiltrated into the matrix, i.e. they did not form a well-developed epithelial layer. However, in the laminated scaffold, keratinocyes formed multi-layers on the surface and fibroblasts were present in the sub-epithelial layer (Fig. 2f).

#### 3.1.8 Electro-spun poly styrene

Electro-spun polystyrene also had good biocompatibility and biostability. It had thicker fibers and larger pore size than the electrospun PLLA. Fibroblasts infiltrated into the pores of the scaffold and keratinocytes grew on the surface (Fig. 2g).

#### 3.1.9 Agarose gel

Keratinocytes did not attach to the fibroblast-populated agarose gel and no epithelial cells were present in the sections after staining.

#### 3.1.10 Collagen-GAG-chitosan porous scaffold

The cross-linked CGC sponge had excellent biocompatibility, bio-stability, and porosity. Normal oral keratinocytes developed a stratified epithelium showing some features of keratinocyte differentiation occurring within the epithelium (Fig. 2h). The TR146 keratinocyte cell line developed a multi-layer stratified epithelium with some differentiation towards the surface to form a non-keratinized epithelium (Fig. 2i). In both cases fibroblasts were present within the scaffold spaces and produced new collagen and other connective tissue components.

### 3.2 Electron microscopy

TEM ultrastructural examination of the engineered oral mucosa produced using normal oral keratinocytes and fibroblasts and the CGC scaffold, confirmed the presence of numerous desmosomal junctions between keratinocytes throughout the epithelium, especially in the spinose layer. It also showed that the amount of cytoplasmic keratin was increased in keratinocytes in the superficial epithelial layers (Fig. 3a and b). Newly synthesized extracellular matrix produced by the fibroblasts was also present in the sub-epithelial layers (Fig. 3c). The appearances were very similar to those of normal oral mucosa.

### 3.3 Immunofluorescence

The results of immunostaining of normal gingival mucosa and the engineered human oral mucosal model (normal oral keratinocytes and fibroblasts on the CGC scaffold) were as follows (Fig. 4):

#### 3.3.1 Cytokeratin 5

There was weak positive staining of basal and mainly suprabasal keratinocytes in normal oral mucosa and to a slightly lesser degree in the engineered oral mucosa.

#### 3.3.2 Cytokeratin 10

There was strong positive staining of keratinizing suprabasal keratinocytes in the upper layers of the normal gingival mucosa and the engineered oral mucosa.

#### 3.3.3 Cytokeratin 19

There was a strong staining of basal, and just supra-basal keratinocytes in the normal gingival mucosa and a similar but slightly weaker pattern of staining in the engineered oral mucosa.

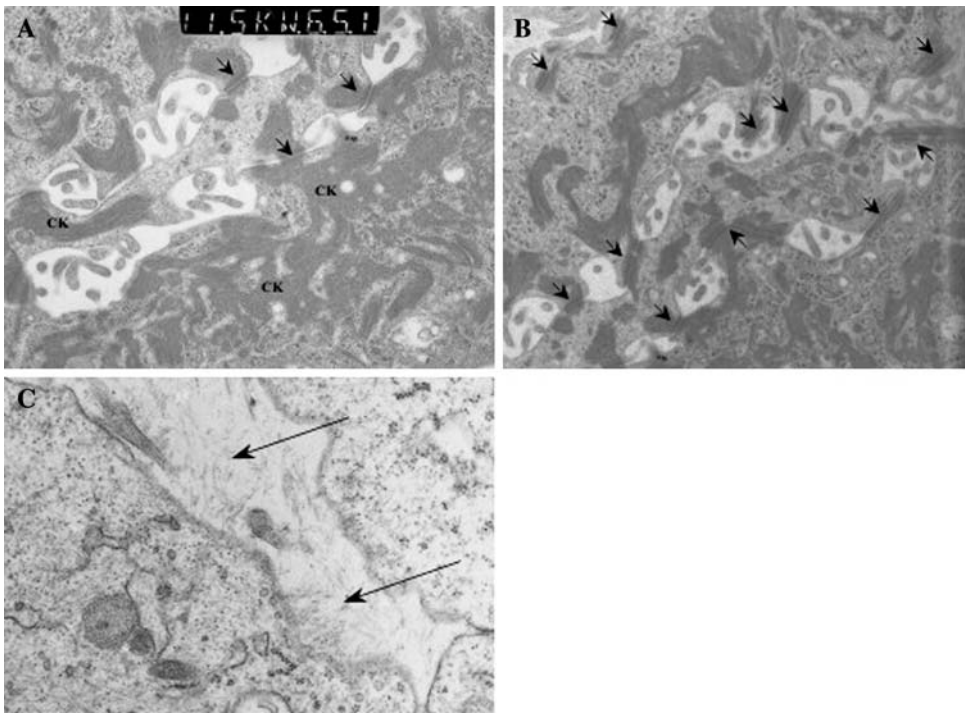


**Fig. 3** TEM pictures of engineered oral mucosa using normal oral keratinocytes and fibroblasts and the CGC scaffold. (**a**) Superficial epithelial layers showing desmosomes (arrowed) and lots of cytoplasmic keratin (CK) (indicated); (**b**) stratum spinosum with numerous desmosomes (arrowed) and (**c**) newly synthesized extracellular matrix in the sub-epithelial layer (arrowed)

*3.3.4 Cytokeratin 13*

There was a negative pattern of staining in both the normal and the engineered oral mucosa.

## 4 Discussion

The suitability of different collagen-based and synthetic scaffolds for producing tissue engineered oral mucosal models was evaluated in terms of biocompatibility, biostability, porosity, and the ability to mimic normal human oral mucosal morphology.

The biostability of the scaffold was an important factor in tissue engineering oral mucosa. The collagen type I membrane had poor biostability compared to the cross-linked collagen and synthetic scaffolds. This confirms that chemical cross-linking with glutaraldehyde is an effective method for modifying the biodegredation rate of collagen-based scaffolds without compromising their biocompatibility [18]. It is important to realize that the ideal biodegradability for a scaffold depends on the proposed application of the engineered oral mucosa. For clinical applications, such as grafting and guided tissue regeneration, a biodegradable scaffold is desirable as this will facilitate its replacement by host tissue, while a non-biodegradable scaffold could induce a foreign body reaction. However, for use as an in vitro test model, the scaffold should have maximum biostability in order to maintain the structural integrity of the model throughout the test procedure.

Another important finding in this study was that the porosity of the scaffold played a crucial role in fibroblast infiltration. With both the collagen-elastin membrane and the collagen bilayer membrane, fibroblasts were unable to migrate through the scaffold due to their lack of porosity. This led to poor fibroblast-keratinocyte interaction and compromised the quality of the engineered mucosa in these cases. Poor fibroblast migration has also been observed using the DED as a scaffold for skin as well as mucosal tissue engineering [8]. Although the porosity of the scaffold is important for fibroblast migration, a porous surface on the epithelial side results in keratinocyte invasion of the scaffold and the formation of epithelial islands within the scaffold. We found that the optimal conditions for encouraging fibroblast infiltration and epithelial stratification, without invasion, could be achieved by laminating a porous scaffold with a collagen-laminin gel on the surface onto which the keratinocytes were to be seeded.

Electro-spun PLLA had a suitable 3D structure for fibroblast ingrowth, however, keratinocytes also infiltrated into non-laminated PLLA scaffolds. It seems that a plain PLLA matrix is suitable for single cell type tissue engineering such as neural tissue [19] or muscle tissue engineering [20]. However, using cell culture inserts it was possible to laminate the scaffold with Matrigel and produce a uniform sheet of the scaffold with controllable size that is very important in terms of accuracy and reproducibility of a test model. Martigel is a basement membrane protein extract that forms a 3D gel at 37 °C and supports cell morphogenesis, differentiation and growth [21]. Its major

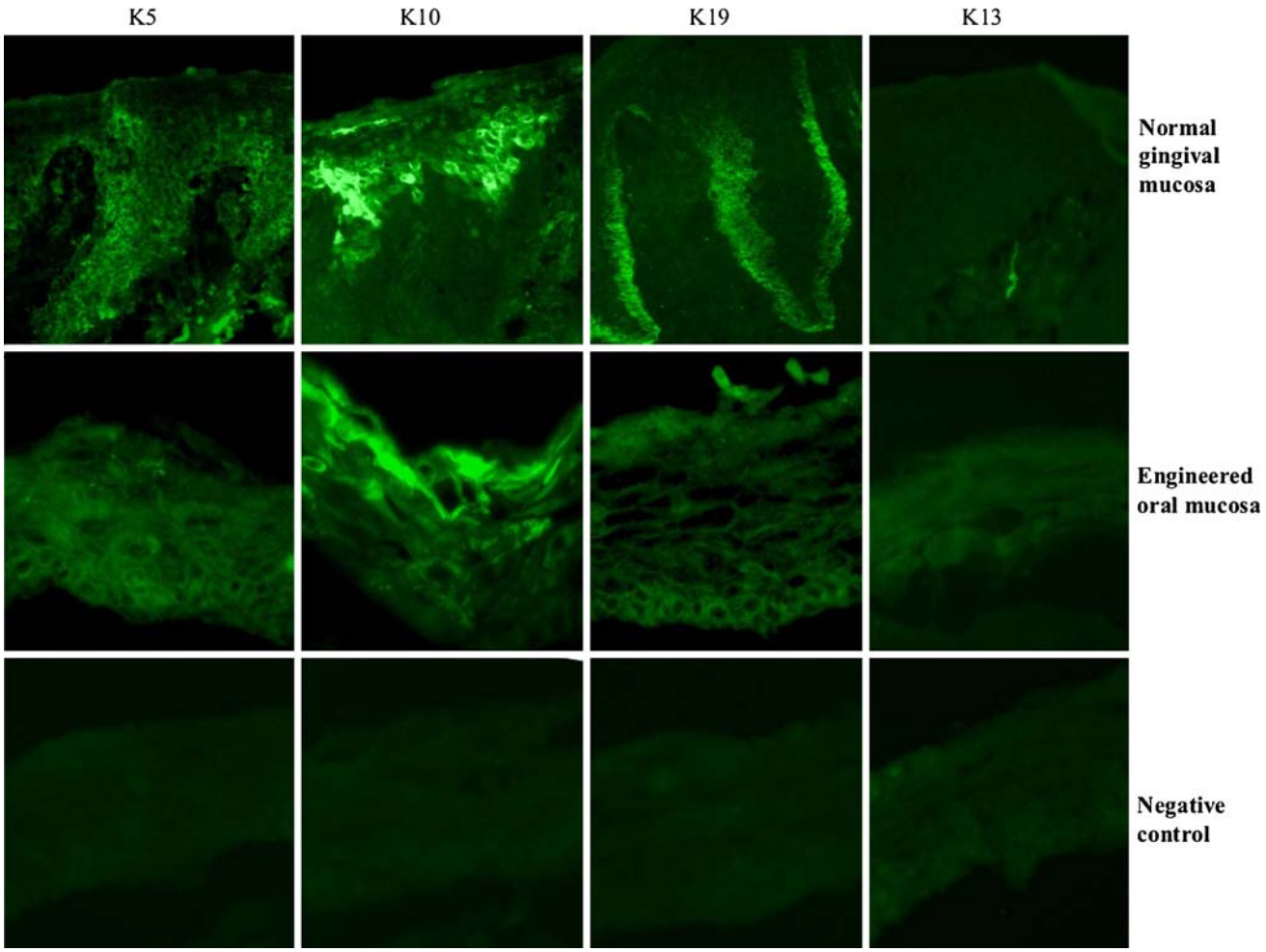


**Fig. 4** Normal gingival mucosa (upper panel) and tissue engineered human oral mucosa (middle panel), using normal oral keratinocytes and fibroblasts on a CGC scaffold, immunofluorescently stained for cytokeratins K5, K10, K19 and K13. Negative controls are shown in the lower panel

components are laminin, collagen IV, heparin sulphate proteoglycans and entactin.

Using the PET and PC membranes, keratinocytes on one side were able to interact with fibroblasts on the other side through the pores of the membrane. The limitation of these systems was the lack of extracellular matrix in the underlying connective tissue layer.

Collagen-chitosan scaffolds have been developed to produce tissue-engineered skin equivalents [22–24]. Chitosan functions as a bridge to increase the cross-linking efficiency of glutaraldehyde due to the longer chain of amino groups. It also accelerates tissue regeneration and fibroblast synthesis of collagen [25]. GAGs, including chondroitin-6-sulphate and hyaluronic acid are hydrophilic components of the extracellular matrix. They attract large amounts of water and form hydrated gels, enabling the rapid diffusion of water-soluble molecules [26].

A collagen type IV-laminin gel (Matrigel) was used to laminate the scaffold surface on which the epithelial cells were to be grown, because it replicates the basement membrane in normal human oral mucosa. In addition, it has been demonstrated that seeding keratinocytes onto a type IV collagen-laminin surface enhances both keratinocyte attachment and migration [27]. In this study the Matrigel coating of the CGC scaffold resulted in superior oral mucosal models that closely replicated the morphology of normal oral mucosa, using both normal keratinocytes and the TR146 cell line. Although TR146 cells do not form a fully differentiated oral epithelium, the need for reproducibility and lack of batch-to-batch variability may make the use of this cell line desirable for in vitro biocompatibility test models.

The use of cell culture inserts, instead of stainless steel rings, was found to be better for the production of engineered oral mucosa, avoided contamination with metal ions and facilitated testing procedures.

Ultra structural analysis of the engineered mucosa identified desmosomes and cytoplasmic keratin in the

spinous and superficial layers of the epithelium with a distribution and morphology similar to normal oral epithelium. In addition, the identification of newly synthesized collagen in the connective tissue compartment indicates that the CGC scaffold is a suitable substrate for oral fibroblasts and promotes extracellular matrix production by these cells. This is consistent with other studies that have demonstrated an increased extracellular matrix production by skin fibroblasts cultured in a CGC scaffold [22, 28].

Immunofluorescence characterization also showed that the cytokeratin expression profile of the optimized engineered oral mucosa was similar to native human gingival mucosa. The cytokeratin profile reflects both the cell type and the differentiation status of keratinocytes within different layers of the epithelium. Cytokeratin 5 (K5) is nromally expressed by basal and supra basal keratinocytes of the oral epithelium. Cytokeratin 19 (K19) is expressed only by basal keratinocytes of the oral mucosa and is a highly specific marker of differentiation. Cytokeratin 10 (K10) is expressed by suprabasal keratinocytes in keratinizing oral mucosa but not in non-keratinizing mucosa. Since the cells originated from the keratinized hard palate, the engineered oral mucosa expressed K10 in the suprabasal layers. Neither, the engineered mucosa nor the normal gingival mucosa (keratinized) expressed K13. This cytokeratin is only expressed by suprabasal cells in non-keratinizing oral epithelium. These finings are indicative of a high level of differentiation within the optimized tissue engineered oral mucosa and show that it accurately reflects the differentiation status and morphology of the tissue of origin.

## 5 Conclusion

In conclusion, biocompatibility, bio-stability, and the porosity of the scaffold are crucial parameters in oral mucosa tissue engineering. Excellent results were obtained using the CGC scaffold for oral mucosa reconstruction. A differentiated and reproducible oral mucosal model was developed that accurately reflects the characteristics of normal oral mucosa and has the potential to be used for biological assessment of dental materials.

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