



Engineering Osteo-Mucosal Constructs: A Review of Adhesion Mechanisms and Innovations

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Article Info	ABSTRACT
Article type: Review	Developing a laboratory-grown construct integrating viable bone and oral mucosal tissues with strong adhesion between the hard and soft tissue layers presents a significant challenge. This engineered tissue construct holds promise as a versatile graft material for diverse oral surgeries and as an in vitro model for evaluating dental materials, dental implants, and oral healthcare products, potentially replacing the need for in vivo studies. Additionally, it offers a valuable tool for investigating various aspects of oral diseases and evaluating the efficacy of different treatments, eliminating the need for animal models. The success of such osteo-mucosal constructs depends on achieving proper adhesion between the bone and connective tissue layers, prompting researchers to conduct studies exploring interlayer adhesion mechanisms. This review provides a comprehensive overview of advances in osteo-mucosal models, conducted through a search of articles published in journals indexed in PubMed, ScienceDirect, Scopus, and Google Scholar databases from January 2010 to January 2024. First, we outline the methods used to construct various components of osteo-mucosal models, including the materials and fabrication techniques. Next, we provide a detailed discussion of the adhesion methods employed. Lastly, we compare the effectiveness of each adhesion method by summarizing the results of cell viability studies, which are crucial for the success of tissue regeneration. This review provides a comprehensive understanding of recent advancements in osteo-mucosal models and their interfacial adhesion, paving the way for promising clinical applications.
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INTRODUCTION

Tissue loss in the orofacial region is a frequent problem that can range from minor periodontal defects to more complicated and challenging defects involving both soft and hard tissues.

Trauma, developmental deformities such as lip and cleft palate, and pathological lesions may affect this area, leading to significant tissue loss and impairments in tissue integrity and continuity, requiring substantial reconstructive

procedures [1]. Autografts are currently considered the gold standard for tissue regeneration. However, they have several disadvantages, such as limited availability, the need for additional surgery for tissue harvesting, difficulty achieving soft tissue closure, and the risk of infection and morbidity at the donor site. Allografts (human grafts other than patient's own tissue) and xenografts (animal grafts from non-human species) also have shortcomings such as the risk of immunological rejection, disease transmission, questionable osteoinductivity particularly in xenografts, blood group incompatibility, and ethical and religious concerns [2]. Due to these limitations, there have been developments in engineered scaffolds and multilayered tissue composites to address these concerns [3-6].

In recent decades, various studies have tried to find suitable materials that can replace specific tissues in the oral cavity, including the periodontal ligament, bone, and oral mucosa [6-11]. Researchers have successfully developed multi-tissue constructs consisting of different components, including scaffolds, diverse cell types, and bioactive molecules to overcome the limitations of clinically available approaches in tissue reconstruction, such as autografts, allografts, and xenografts [1,10]. However, creating an integrated functional structure that provides a suitable environment for the proliferation, distribution, and differentiation of various cell types remains a challenge. Hence, researchers such as Tabatabaei et al, [3] Sun et al, [12] and Almela et al. [13] have focused on developing engineered complex tissue models such as osteo-mucosal constructs to address the current issues. Reaching the maximum possible tissue regeneration within the oral and maxillofacial region requires an ideally designed three-dimensional [14] structure with appropriate mechanical and chemical features supporting cell proliferation and differentiation [15,16]. Despite progress in this field, achieving optimal interlayer adhesion in terms of biocompatibility, biodegradability, and mechanical strength remains a challenge [3,17-19].

The interface between soft and hard tissues in a physiological setting serves as a connector between disparate tissues, playing a key role in maintaining tissue integrity. Epithelial cells in the soft tissue connect to a basement membrane via hemidesmosomes, which provide a connective tissue interface to the lamina propria. The lamina propria facilitates both nutrient exchange and mechanical anchorage, comprising an elastin and collagen matrix, vasculature, nerves, and various cells. Beneath the lamina propria is the submucosa, which also contains collagenous/elastic matrix, nerves, and vasculature [20]. Given the complexity of cellular and matrix composition, achieving an optimal substitution for this layer is still challenging, despite significant advances in engineered tissue constructs [21]. Soft and hard tissue constructs can be joined using various methods. One possible method for this purpose is physical adhesion, which combines heat or solvent treatment with press fitting. However, this method can hinder cell proliferation in the affected area. Suturing is another potential method for adhesion, but it may not always be applicable in engineered soft tissues where some hydrogel-based scaffolds may have low tear strength [3,22,23]. Adhesives are another option for joining tissues. Adhesives can be classified into two categories: synthetic/semi-synthetic and biologically-derived adhesives [24,25]. Cyanoacrylate and fibrin glue are two examples of commercial adhesives. Cyanoacrylate is a synthetic/semi-synthetic adhesive that provides strong adhesion to tissue. Fibrin glue, on the other hand, is a biologically-derived adhesive that rapidly forms a stable clot. These adhesives are primarily used for hemostasis; however, they have some disadvantages such as the toxic byproducts formed by cyanoacrylate degradation and the low adhesive strength of fibrin glue [26]. Moreover, presence of an adhesive layer may disrupt intercellular communication between different layers [27]. Therefore, some researchers, such as Tabatabaei et al. [3] explored methods to directly integrate soft and hard constructs without the need for a third material.

A limited number of studies by Tabatabaei et al, [3] Almela et al, [13] Fuchs et al, [28] Bae et al, [29] and Almela et al. [30] have investigated the engineered osteo-mucosal models with an optimized soft/hard tissue adhesion. Therefore, the purpose of this review was to evaluate studies focusing on engineering of osteo-mucosal models simulating the anatomical structure of the oral cavity.

MATERIALS AND METHODS

An electronic search was conducted for relevant articles published in journals indexed in PubMed, ScienceDirect, Scopus, and Google Scholar databases from January 2010 to January 2024. The methods used to construct various components of osteo-mucosal models, including the materials and fabrication techniques were outlined and the effectiveness of each adhesion method was assessed by summarizing the results of cell viability studies, which are crucial for the success of tissue regeneration.

RESULTS AND DISCUSSION

The current osteo-mucosal designs struggle to achieve an optimal integration between hard and soft tissues, which is crucial for successful tissue regeneration (Fig. 1). A summary of relevant studies investigating engineered osteo-mucosal constructs is provided in Table 1. In this review, we describe the fabrication of osteo-mucosal models in a classified manner, based on the included studies, as follows:

Isolation and culture of cells

Bae et al. [29] pioneered the use of osteo-mucosal models to investigate the adverse effects of bisphosphonates. They cultured immortalized human oral keratinocytes by the human papillomavirus-16 (HOK-16B), normal human oral keratinocytes (NHOKs), and normal human oral fibroblasts (NHOFs) obtained from healthy biopsy samples.

In another osteo-mucosal-based study, Almela et al. [30] cultured three different cell types: rat osteosarcoma-derived cell line (ROS cells), which exhibits a typical osteoblastic phenotype and displays responses similar to normal bone cells [22], an immortalized

human oral epithelial cell line (OKF6/TERET-2), and NHOFs derived from keratinized gingiva biopsy samples. It should be noted that ROS viability decreased during the second and third months of the experiment due to their high demand for nutrients to support fast-paced proliferation.

Almela et al. [13] developed a fully characterized osteo-mucosal model based on primary human cells in a similar study. They isolated NHOKs and NHOFs from the epithelial and connective tissue layers of gingival biopsy samples. They substituted ROS cell line with isolated human alveolar osteoblasts (HAOBs) from sterile bone chips obtained during dental implantation surgery. This change was made to address the decreased viability of ROS in their previous experiment [30].

In another study, Almela et al, [14] also developed an osteo-mucosal model based on the collection and isolation of NHOKs and NHOFs derived from gingival biopsy samples, and primary HAOBs derived from waste bone chips of patients receiving osteotomy for implant placement, similar to their previous study [13]. All different cell types were then cultured in complete Dulbecco's modified Eagle's medium (DMEM) and left undisturbed for 7 days to prevent the possible impact of dislodgment on cell proliferation.

To synthesize the engineered multilayered



Fig. 1. Schematic representation of engineered construct combining functional bone and oral mucosa.

Table 1. Summarized information about engineering osteo-mucosal constructs in relevant studies

Authors	Cells	Scaffold	Scaffold fabrication method	Adhesion	Significant features and results
Tabatabaei et al, [3]	OSs, OFs, OKs	PCL β-TCP	Solvent casting/ evaporation technique	Michael additions	Tyrosinase-treated group: no cytotoxicity, lower cell proliferation at 7 and 14 days after cell culture, better cell distribution after 2 weeks, more star-shaped osteocytes and hydroxyapatite dendritic-shaped crystals, higher expression of calcium and phosphate Completely developed epithelium, connective tissue, and hard tissue within the integrated model with slightly thinner epithelium than the soft tissue-only models Adhesion assessment: cohesive debonding in integrated model and adhesive debonding in control group Shear strength: integrated model (157.6±25.1 KDa), control group (149.4±23.1 KDa)
Fuchs et al, [28]	MG63, HaCaT, L929	PCL	MEW	Heat sealing	Increased viability of MG63 cells implanted in both box-shaped scaffolds with 250 and 500 μm fiber spacing, HaCaT cells implanted in triangular-shaped scaffolds with 250 μm fiber spacing, and L929 cells implanted in triangular-shaped scaffolds with 250 μm fiber spacing during the 28 days of the experiment with still lower viability compared to the control Increased viability of all cell types in bilayered core/surface fusion products compared to the separated models
Almela et al, [14]	NHOKs, NHOFs, HAObS	β-TCP	3D printing	Biocompatible fibrin-based adhesive sealant (ARTISS)	OMMs: a well-developed, multi-layered oral epithelium mimicking NOM. CK13 was strongly expressed throughout the entire epithelium, while CK14 was strongly expressed only in the basal layer. Bone scaffold: macroporous structure provided cell adhesion, penetration, and proliferation on both sides. Cell proliferation occurred along scaffold strands, and the compact side was covered by a cellular layer. Inter-tissue adhesion was similar to the normal oral osteo-mucosal tissues. ABMM, showed the highest expression of COL1 and ON.

Table 1 Cont'd

Almela et al, [13]	NHOKs, NHOFs, HAOBs	HA/ros TCP	Not mentioned	Biocompatible fibrin-based adhesive sealant (ARTISS)	Vital HAOBs, NHOFs, and NHOKs during 17 days of cultivation Differentiated stratified epithelium, fibroblast populated connective tissue, and bone structures of ABMM similar to the natural tissue Scaffold pores filled with secreted matrix and viable cells Sub-epithelial layer demonstrated newly formed collagen Gene expression of the composite model: COL1 (highest), ALP, OC, ON, OP
Almela et al, [30]	ROS, OKF6/TERET-2, NOF	HA/TCP	Not mentioned	Biocompatible fibrin-based adhesive sealant (ARTISS)	Decreased metabolic activity and density of ROS at the end of 1st and 3rd months Reduction in cellularity over the experiment time 1st month: Cell proliferation within the scaffold 2nd month: accumulation of calcified nodules 3rd month: accumulation of minerals Higher portion of closed porosity in the EB model in comparison to the HA-TCP scaffold, leading to higher surface density
Bae et al, [29]	HOK-16B, NHOKs, NHOFs	Neonatal rats' calvaria	Natural bone	Direct placement	The osteo-mucosal model consisted of well-differentiated epithelial and bone tissues, and a periosteum-like layer similar to the native tissue

OSs: oral osteoblast; OFs: oral fibroblasts; OKs: oral keratinocytes; NHOKs: normal human oral keratinocytes; NHOFs: normal human oral fibroblasts; HAOBs: human alveolar osteoblasts; HA/TCP: hydroxyapatite/tricalcium phosphate; MEW: melt electrospinning writing; OMMs: oral mucosal models; NOM: normal oral mucosa; CK: cytokeratin; ABMM: alveolar bone and oral mucosal model, COL1: collagen 1, ON: osteonectin; NOF: normal oral fibroblasts; EB: engineered bone, ALP: alkaline phosphatase; OC: osteocalcin; OP: osteopontin; β -TCP: B-tricalcium phosphate; MG63: human osteoblast-like cell line; HaCaT: keratinocyte cell line; L929: fibroblast cell line; OKF6/TERET-2: immortalized human oral epithelial cell line; ros: rat osteosarcoma-derived cell line;

membrane with oral hard and soft tissues, Fuchs et al. [28] cultivated MG63 human osteoblast-like cell line, HaCaT keratinocyte cell line, and L929 fibroblast cell line in DMEM. Tabatabaei et al. [3] derived oral osteoblasts, fibroblasts, and keratinocytes from gingival and bone biopsy samples taken from healthy individuals undergoing oral surgery in their *in situ* study. After isolation and characterization, all three different cell types were co-cultured in Green's medium, which was found to be the most balanced medium for oral osteoblasts, fibroblasts, and keratinocytes based on PrestoBlue® assay results investigating cell attachment and proliferation. This medium consisted of DMEM/F12 (3:1), 10% fetal bovine serum (Sigma, USA), 1% antibiotic/antimycotic (Sigma, USA), 5–10ng/mL epithelial growth factor, 5–10µg/mL insulin, 0.025µg/mL adenine, 0.5µg/mL hydrocortisone, 5µg/mL transferrin, and 1.3ng/mL triiodothyronine. Among all the evaluated co-culture media in this study, Green's medium demonstrated a significantly higher proliferation of oral keratinocytes, while oral fibroblasts and oral osteoblasts were adaptable in almost all assessed media.

Fabrication and characterization of hard tissue scaffolds

Developing a scaffold that mimics the natural cortico-cancellous bone structure, which is a composite of organic and mineral materials, is the initial stage of compound tissue reconstruction [31].

Bae et al. [29] used calvariae taken from neonatal rats as their hard tissue construct. However, the use of xenografts can increase the risk of disease transmission [3]. More recent studies have instead fabricated hard tissue scaffolds to use as their hard tissue component. For example, Almela et al. [30] synthesized ceramic discs made of hydroxyapatite/tricalcium phosphate (TCP) (60%/40%) with a pore diameter of 200–450µm for use as scaffolds. TCP is widely utilized in dental regenerative procedures due to its favorable degradability, osteoconduction, and osteoinduction. The mixture of hydroxyapatite/TCP has been shown to work synergistically, maintaining the volume of the

grafted site, and replacing bone tissue appropriately [32]. ROS cells were then seeded dropwise onto the scaffolds and allowed to adhere for 2 hours. The cell-included scaffolds were cultured for 3 months in high-glucose complete DMEM.

In a similar study, Almela et al. [13] used cultured HAObBs in complete DMEM with additional L-ascorbic acid 2-phosphate to create the hard tissue component of their model. To achieve this, they implanted the cells dropwise into ceramic disc scaffolds made of hydroxyapatite/TCP (60%/40%), similar to their previous study [30], which had 78.9% porosity. The HAObBs were left undisturbed for 2 hours to attach to the scaffolds. After 24 hours of coverage of the models with complete DMEM with additional L-ascorbic acid 2-phosphate, they were immersed in the medium for the next 17 days. Almela et al. [14] also developed an osteo-mucosal model in another study using a 3D-printed scaffold, similar to their previous study [13]. The scaffold was disc-shaped and was fabricated through 3D bioprinting of an injectable β -TCP paste. The scaffolds were then air-dried, sintered, autoclaved, and pre-wetted. Osteoblasts were then seeded in a dropwise manner. After culture, which was conducted in complete DMEM/ascorbic acid, the cell-scaffold discs were suspended for 17 days while spinning (30rpm).

The scaffolds used in the study conducted by Fuchs et al. [28] were made of medical-grade poly(ϵ -caprolactone) and were fabricated using melt electrospinning writing (MEW). This innovative method combines melt electrospinning with 3D printing, enabling precise placement of fibers. As a result, it allows for the construction of structures with greater accuracy and reproducibility compared to traditional 3D printing methods [33]. They used melted polymers heated up to 73.0°C. This unique 3D printing technology enables fabrication of well-defined fiber scaffolds and has been widely used to design tissue-engineered scaffolds using melted polymers [34]. MEW has addressed the drawbacks of using solvents, such as generation of toxic substances, by using the melting technique

[35]. Box-shaped surface scaffolds were designed with fibers measuring 20µm in diameter, spaced 250 or 500µm apart, and MG63 cells were implanted into the scaffold. Tabatabaei et al. [3] printed 3D ceramic/polymer hard tissue scaffolds consisting of polycaprolactone (PCL) and β -TCP. PCL/ β -TCP scaffolds are among the most widely used materials for bone regeneration [36]. The scaffold was strengthened with PCL, a hydrophobic polyester, providing enhanced mechanical properties [37]. Despite the poor mechanical properties of β -TCP [38], addition of this bioceramic component improved osteoconductivity for osteoblast differentiation in the scaffold. The addition of β -TCP to PCL also resulted in a significant decrease in contact angle and an increase in water sorption. PCL/ β -TCP scaffolds were fabricated using the solvent casting/evaporation technique, enabling the application of ultra-thin layers on the desired surface, followed by hot melt printing [39]. The discs were printed layer by layer in a gradient confirmation of pore size from one side to the other, with a 15mm diameter, 4mm thickness, and 250µm strand size. The pattern of intervals between the strands was gradually increased from 0.8mm on one side to 1.40mm on the other side.

Construction of engineered soft tissue model

Different approaches have been utilized in the literature for the fabrication of soft tissue components in osteo-mucosal models. For instance, Bae et al. [29] used an acellular collagen matrix and seeded NHOFs cultured in DMEM/199 within the matrix using the raft culture system. The researchers placed calvaria obtained from neonatal rats as a scaffold on top of the acellular collagen matrix. The raft system utilizes a 3D tissue culture that enables the differentiation of the top layer, specifically the keratinocyte layer, on collagen gels via the air-liquid interface [40]. After solidification of NHOFs in the matrix for 3 days, HOK-16Bs were added to the top of the construct and allowed to proliferate over the next 4 days. During the next week, keratinocytes were differentiated by air-lifting the collagen/NHOFs/HOK-16Bs matrix within the Transwell insert.

Almela et al. [30] fabricated their oral

mucosal model using rat-tail type I collagen obtained from Wistar rats. They prepared and neutralized the collagen-based solution, then added primary fibroblasts and allowed it to solidify for 2 hours. After 3 days of culture, the gel contracted, and OKF6/TERET-2 cells were seeded on it, allowing them to adhere for 2 hours. Once the epithelial cells covered the adherent surface of the gel, the construct was cultured through an air/liquid interface for 12-14 days.

In their subsequent study, Almela et al. [13] created oral mucosal models using collagen-based scaffolds that contained rat-tail type I collagen. They employed the technique first introduced by Dongari Bagtzoglou and Kashleva [41]. To build the model, they seeded NHOFs, which were air/liquid interface-cultured for 10 days on the collagen scaffold. According to the evaluations, this synthesized model featured a differentiated basal layer and epithelial layer, with 12-13 distinct NHOKs that secreted cytokeratin, resembling natural epithelium. Similarly, Almela et al. [14] constructed oral mucosal models based on the Dongari Bagtzoglou and Kashleva [41] technique, which was previously explained.

To design the soft tissue component, Fuchs et al. [28] implanted HaCaT and L929 cell lines into triangular-shaped scaffolds made with melted PCL. To create the scaffolds, they filled a prepared stainless-steel slide with melted PCL granulates heated at 73.0°C until a glazed surface formed due to the fusion of granules. These scaffolds were similar to their box-shaped scaffolds described previously in hard tissue fabrication, with 20µm diameter fibers and a spacing of 250µm between them. As an innovative part, they synthesized bacteria-tight barrier membranes through film casting.

In the study conducted by Tabatabaei et al, [3] the soft-tissue component was directly synthesized on top of the hard tissue scaffold. The fabrication method for the soft tissue is described in the next section.

Soft/hard tissue adhesion

A functional construct that combines bone and mucosal tissues needs a strong interfacial adhesion that can connect the two distinct structures without limiting cellular

proliferation and differentiation.

The osteo-mucosal tissue construct developed by Bae et al. [29] involved placing rats' calvaria directly on an acellular collagen matrix, followed by the addition of NHOFs and HOK-16B cells. The constructs were then air-lifted for 2 weeks before being harvested. Although the bonding strength of the soft/hard tissue interface was not measured, the study revealed that the placement of the calvarial bone between the epithelium layer and the medium did not impede the delivery of appropriate nutrients, as indicated by K14 staining results. Almela et al. [30] combined fabricated hard and soft tissue models using a biocompatible fibrin-based adhesive sealant (ARTISS, Baxter, UK). A thin layer of the mixed protein-thrombin sealer was applied on the soft tissue models' dermal surface. It was then held for a minimum of 3 minutes with gentle pressure to ensure proper adhesion between the layers. The sealant (ARTISS, Baxter, UK) was completely set, and the composite construct was then cultured at an air/liquid interface for an additional 5 days before undergoing histological examination. The histological assessment of the bone-oral mucosa construct revealed parakeratinized epithelium consisting of 7-9 OKF6/TRET-2 layers, equally distributed fibroblasts in the connective tissue, a thin adhesive layer, and distributed ROS beneath the sealer layer. The authors explained that they produced the hard tissue and soft tissue components of their model separately because there was no universal medium that could support cell viability for a long period of time.

In another study, Almela et al. [13] established an adhesion between the soft tissue and hard tissue components using a biocompatible fibrin-based adhesive sealant (ARTISS, Baxter, UK). After 17 days of tissue culture in both components, a thin layer of this mixed fibrinogen-thrombin sealer was applied on the non-epithelial side of the oral mucosal model and it was placed in a favorable position on the surface of the bone model. Gentle pressure was applied for at least 60 seconds, which was less than that in their previous study [30], where this mechanism was used for adhesion. The constructs were then cultured for an

additional 5 days. The bone and the oral mucosal model of the subsequent study by Almela et al. [14] were adhered with a fibrin adhesive sealant (ARTISS, Baxter, UK) in the same manner as in their former study [13].

To fuse the two components of surface scaffolds and core membranes, Fuchs et al. [28] used heat sealing, without the need for an additional adhesive material. In this method, core membranes were heated on a steel slide up to 70°C until they were glazed. While the membranes were cooling, the scaffolds were placed over them and instantly chilled using cold deionized water.

In a recent study regarding osteo-mucosal models by Tabatabaei et al, [3] an interfacial adhesion technique was employed using a two-step biocompatible chemical reaction based on the Michael additions to eliminate the need for an adhesive layer. This technique involved the direct formation of soft tissue on the surface of functionalized hard tissue scaffolds through a reaction between 1,6-hexanediamine (HDA) and tyrosinase in the hard and soft tissues, respectively. The 10wt% HDA was used in this experiment as it had a positive effect to enhance cell proliferation on the surface structures. The hard tissue scaffolds were made of PCL/TCP, and the amino group (-NH₂) present in HDA was introduced to the scaffold. The aminolysis reaction between the amino group (-NH₂) of HDA and the ester group (-COO-) of PCL led to the formation of an amide linkage (-CONH-), which transformed the PCL/TCP into a modified scaffold (mPCL/TCP) [42]. In the next step, the encapsulated osteoblasts in collagen were injected and immobilized into the mPCL/TCP scaffold through the Michael reaction. Tyrosinase, as a cross-linking enzyme, was added to the collagen for functionalization purposes (mPCL/TCP/Col/Ty) as it also increases cell viability [43]. Tyrosinase oxidized the phenol content of tyrosine, a residual component of collagen, through diphenolase activity, producing dihydroxyphenylalanine (DOPA) and DOPA-quinone. DOPA-quinone is a highly reactive compound that can be transformed into DOPA or participate in the Michael additions in corporation with amino groups

forming cross-links [44]. Engineered lamina propria was added on top of the mPCL/TCP/Col/Ty by embedding oral fibroblasts in a collagen hydrogel. The oral mucosa was directly synthesized on the surface of the mPCL/TCP/Col/Ty scaffold by seeding oral keratinocytes. In contrast to the experimental group, the soft and hard tissues were fabricated separately in the control group. In this regard, the non-functionalized hard tissue scaffold and the fabricated oral mucosa consisting of oral keratinocytes were adhered through cyanoacrylate oral adhesive following the manufacturer's instructions. Based on the results of hematoxylin and eosin staining, all the epithelium, connective tissue, and hard tissue components were developed in this integrated model. However, the epithelium layer was slightly thinner than that in the control soft tissue-only models.

According to the results of hematoxylin and eosin staining, osteoblast cells were well-distributed in tyrosinase-treated scaffolds, which could be attributed to the cross-linking property of the scaffold. Furthermore, the higher expression of calcium and phosphate, as well as the presence of more star-shaped cells resembling osteocytes confirmed the differentiation of osteoblasts. This observation could explain the decrease in cell proliferation after 7 days, as they had already undergone differentiation [45].

Tabatabaei et al. [3] employed DOPA-functionalization technique, a bioinspired strategy based on mussel adhesion. This approach addressed issues such as swelling, decreased bond strength, and instability in wet environments, which are the limitations of conventional adhesives according to a study by Ge and Chen [46]. Adding aminolysis and tyrosinase to the hard tissue component caused strong bonding between hard and soft tissue constructs with deposited extracellular matrix from osteoblasts and fibroblasts. However, the authors suggest that further studies are required to understand the long-term behavior of this model after implantation. For this purpose, it is necessary to increase the thickness of the epithelium layer for adequate vascularization, which is possible by using a co-culturing system

of endothelial cells with fibroblasts or osteoblasts to facilitate nutrient delivery. They also recommended using a bioprinting approach with bio-ink-containing cells, as it can lead to improved cell distribution and deposition compared to acellular scaffold printing.

Cell viability

Several types of assays can be used to determine cell viability, indicating the number of healthy cells or the rate of proliferation in a construct, and each assay has its advantages and drawbacks [47,48]. Some available cell viability assays based on the detection method are colorimetric assays, including thiazolyl blue tetrazolium bromide (MTT) and water-soluble tetrazolium assays, fluorometric assays such as resazurin, or more recent resazurin-based assays such as the PrestoBlue (PB) assay. Although MTT is one of the most commonly used assays for cell viability, it may overestimate or underestimate cell viability [47]. The PB assay has gained popularity due to its high sensitivity in measuring cellular metabolism while being safe, flexible in measurement method, time-saving, and simple in utilization through fluorescence detection [49]. This method involves one single step with a recommended incubation time of 10 minutes to 2 hours and a sensitivity of 12 cells/well; whereas the MTT assay is a two-step method with a recommended incubation time of 2 hours and a sensitivity of 1000 cells/well [50]. According to a study conducted by Boncler et al, [50] the PB assay could be an appropriate alternative to the MTT assay as a fast, simple, and continuous monitoring and analysis method. To assess cell viability, Bae et al. [29] used the MTT assay after cell treatment. However, they did not conduct this assessment to evaluate the cell viability of osteo-mucosal tissue construct, which is the focus of this study. Almela et al. [30] assessed cell viability utilizing the PB assay (Invitrogen, USA). The results showed a significant decrease in cell viability after 3 months. They suggested that this decrease may be due to the following reasons: 1) the spinner bioreactor enhances the transport of external mass, 2) the formation of extracellular matrix on the surface of osteo-mucosal model during the

extended culture period might have prevented nutrient transport through diffusion, and 3) the use of ROS cells, which have a high proliferation rate and therefore require excessive nutrient supply.

In another study, Almela et al. [13] used the PB assay, which demonstrated that all the HAOBs, NHOFs, and NHOKs remained viable during the short duration of the experiment. They discussed that the oral mucosal models had no difficulty with oxygen and nutrient transport in a static culture due to the hydrophilic nature of their collagen hydrogel. However, when hard and soft tissues were combined, the ceramic-based bone model prevented oxygen and nutrient transport through the increased layers of the construct. As a result, the experiment only lasted for 5 days. In a similar study conducted by Almela et al, [14] the fabricated osteo-mucosal model was assessed utilizing histological observation; however, they did not evaluate cell viability. This assessment in a study by Fuchs et al. [28] was carried out using the water-soluble tetrazolium assay, a screening technique to assess cell vitality by measuring proliferation and cytotoxicity through mitochondrial dehydrogenase activity [51]. The result revealed a 25% increase in osteoblast cell viability, while keratinocytes had a 5% decrease in cell viability. In addition, bilayers in this study demonstrated higher cell viability in comparison with core membranes or surface scaffolds, which can be related to the heat and pressure applied for interconnectivity between the construct's polymer fiber layers.

Tabatabaei et al. [3] measured cell viability in different stages of the experiment with the utilization of the PB assay. The results of the PB assay after co-culture of oral fibroblasts, osteoblasts, and keratinocytes revealed that all three types of cells were most adaptable to Green's medium. The PB assay results supported high initial attachment and proliferation of cells on PCL/TCP60 scaffolds. After incorporation of osteoblasts into the hard tissue scaffold and adding tyrosinase, a doping agent that

promotes cell viability through providing cross-linking [43], PB assay results after 72 hours, and 7 and 14 days revealed no cytotoxicity within the first 72 hours, followed by a significant decrease in cell proliferation after 7 and 14 days. The presence of osteocyte-like cells with calcium and phosphate expression in the tyrosinase-treated group suggested differentiation of osteoblasts, which also explained the reduction in cell proliferation after 7 days, as differentiation leads to lower proliferation.

CONCLUSION AND FUTURE PERSPECTIVE

Osteo-mucosal models can be applied in various conditions. Such models aim to develop a 3D bioengineered structure that can effectively replace large, damaged areas, which is particularly challenging when integrating different types of tissues, such as bone and mucosal tissues. Besides complex tissue reconstruction, they can be used as an alternative to animal models and as a system for controlled drug delivery. Additionally, they can be utilized as a modeling tool for oral diseases, such as bone invasion associated with oral cancer.

The application of currently available adhesives in clinical situations is limited due to their low biodegradability, biocompatibility, and availability, as well as high cost, which can compromise the direct integrity of layers. Fibrin-based sealants, an example of such adhesives, have gained popularity for surgical purposes due to their biocompatibility. However, their biodegradability could negatively affect tissue reconstruction. Therefore, further studies investigating fibrin-based sealants in clinical situations are required. On the other hand, introducing Michael addition for interlayer adhesion could enable long-lasting adhesion, supporting adequate cell proliferation. Despite significant advances in this field, further research is necessary to fully explore the clinical applications of these constructs.

There is still limited literature available on osteo-mucosal designs with optimal interlayer adhesion. An ideal adhesive

should meet the following requirements for optimal efficiency: (I) excellent biocompatibility providing cellular adhesion and proliferation; (II) ample mechanical strength to sufficiently bear external loads, particularly in extensive tissue reconstructions; (III) allowing interlayer transportation of nutrients and essential substances; (IV) suitable biodegradability providing space for newly constructed tissue, and (V) applicability in various clinical situations.

Further research is needed to investigate the capability of the adhesion for interlayer transportation of nutrients and oxygen supply, cell survival, particularly in thicker layers, loading and delivery methods, and clinical applications. While only a few in vitro studies have investigated applicable interlayer adhesion, further progress in the clinical performance of osteo-mucosal designs will be crucial to advance the field. In addition, clinical studies would provide valuable information about the interactions between environmental factors and components of osteo-mucosal models, in comparison to potentially controversial and misleading in vivo research.

Developing an optimal co-culture medium that supports the prolonged cultivation of all oral keratinocytes, oral fibroblasts, and oral osteoblasts is another aspect of osteo-mucosal models that requires further investigation. Such a medium should provide a balanced environment for all three cell types, support optimized cellular proliferation, differentiation, and intercellular communication, and maintain function for a long period of time. Future studies may involve optimizing culture conditions using different bioreactors to enhance tissue growth and incorporating vascularization to maintain the tissues' viability in the long term.

CONFLICT OF INTEREST STATEMENT

None declared.

GENERATIVE AI IN SCIENTIFIC WRITING

None declared.

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