The Reconstruction of the Physiological Environment for Growing In-Vitro Human Salivary Organoids

By

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List of Abbreviations*

ANOVA	Analysis of variance
APFECC	Animal Product-free Epithelial Cell Culture
BPE	Bovine pituitary extract
CalAM	Calcein acetoxymethyl
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F-12	50:50 Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient Mix
EGF	Epidermal growth factor
EthD-III	Ethidium Homodimer III
EW	Egg white
EWA	Egg White Alginate
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
HNC	Head and neck cancer
KGM-Gold	Keratinocyte Growth Media – Gold
MEM	Minimum Essential Media
MEM-α	Minimum Essential Media Alpha
MTT	Thiazolyl blue tetrazolium bromide
NS-SV-AC	Normal salivary simian 40-immortalized acinar cell
PBS	Phosphate-buffered saline
SE	Standard Error
SMG-hu-1	submandibular gland-human-1
SS	Sjögren's Syndrome
TERM	Tissue engineering and regenerative medicine

* Includes only relevant abbreviations and those that appear 3 times or more in-text

Abstract

Introduction: Salivary glands and their function are crucial to the maintenance of good oral hygiene and health. The loss of such functions results in a condition known as xerostomia which can lead to further complications such as difficulty in speech, mastication, and increased susceptibility to dental caries and oral infections and diseases. Annually, approximately 900,000 new patients risk these complications due hypo- or dysfunctional salivary function from head and neck cancer (HNC) radiotherapy or the onset of Sjögren's Syndrome (SS). While some palliative treatments are available for patients suffering with xerostomia, there are no curative treatments to date.

Objectives: This project addresses this issue by attempting to optimize the growing conditions such as the culture media and bio-scaffold for salivary glands grown in 3D. These 3D glands (organoids) can then be used for studies in support of a curative treatment for xerostomia such as drug therapy testing or disease modeling. This project is a pilot study which establishes and observes the potential of a novel animal-product free media, and an Egg White Alginate (EWA) scaffold for culturing salivary organoids.

Methods: NS-SV-AC and SMG-hu-1 cell line proliferation was measured and compared across Animal Product-free Epithelial Cell Culture (APFECC), Epi Max, and Keratinocyte Growth Media – Gold (KGM-Gold) using an MTT assay over eight days. Cell viability of both NS-SV-AC and SMG-hu-1 cell line was observed over 20 days and 10 days using a Live-Dead stain and AlamarBlue assay respectively.

Results: The results indicated that the novel animal product-free media, APFECC growth media, was not significantly different from Epi Max and KGM-Gold; APFECC growth media's ability to induce cell proliferation was evident. Results for the EWA scaffold suggested that it is an excellent candidate for salivary organoid as it can sustain cell survivability in two salivary cell lines—NS-SV-AC and SMG-hu-1—however it is unclear if proliferation was present.

Conclusion: While it was evident that APFECC growth media can support cells, further studies need to be conducted to reoptimize the growth media. Similarly, while EWA can support

salivary cells grown in 3D even after 20 days, further studies need to be carried out to determine if there were any phenotypic changes in the cell culture, and also to further characterize the scaffold. Both products should be tested with human primary salivary cells to truly test its potential. Regardless, the results found here provide new insights and tools for researchers to culture salivary cells in both 2D and 3D depending on their goals.

Résumé

Introduction: Les glandes salivaires et leur fonction sont essentielles au maintien d'une bonne hygiène de santé bucco-dentaire. La perte de ces fonctions entraîne une maladie appelée xérostomie qui peut entraîner d'autres complications telles que des difficultés d'élocution, de mastication, et d'une susceptibilité accrue aux caries dentaires et aux infections et maladies bucco-dentaires. Chaque année, environ 900 000 nouveaux patients risquent ces complications en raison de la fonction salivaire hypo- ou dysfonctionnelle de la radiothérapie pour le cancer de la tête et du cou ou de l'apparition du syndrome de Sjögren (SS). Bien que certains traitements palliatifs soient disponibles pour les patients souffrant de xérostomie, il n'existe aucun traitement curatif à ce jour.

Objectifs: Ce projet s'attaque à ce problème en tentant d'optimiser les conditions de croissance telles que le milieu de culture et l'échafaudage biologique pour les glandes salivaires cultivées en 3D. Ces glandes 3D (organoïdes) peuvent ensuite être utilisées pour des études à l'appui d'un traitement curatif de la xérostomie, tel que les tests de pharmacothérapie ou la modélisation de maladies. Ce projet est une étude pilote qui établit et observe le potentiel d'un nouveau milieu exempt de produits d'origine animale et d'un échafaudage en alginate de blanc d'œuf (EWA) pour la culture d'organoïdes salivaires.

Méthodes: La prolifération des lignées cellulaires NS-SV-AC et SMG-hu-1 a été mesurée et comparée sur des cultures de cellules épithéliales sans produit animal (APFECC), Epi Max et des milieux de croissance kératinocytaire - Or (KGM-Gold) à l'aide d'un dosage au MTT sur huit jours. La viabilité cellulaire des lignées cellulaires NS-SV-AC et SMG-hu-1 a été observée sur 20 jours et 10 jours en utilisant une coloration Live-Dead et le test AlamarBlue, respectivement.

Résultats: Les résultats ont indiqué que le nouveau milieu sans produit animal, le milieu de croissance APFECC, n'était pas significativement différent des milieux de croissance Epi Max et KGM-Gold; la capacité du milieu de croissance APFECC à induire une prolifération cellulaire était évident. Les résultats d'échafaudage EWA suggèrent qu'il s'agit d'un excellent candidat pour les organoïdes salivaires, car il permet de maintenir la survie des cellules dans deux lignées

cellulaires salivaires - NS-SV-AC et SMG-hu-1 -, mais il n’est pas clair si la prolifération était présente.

Conclusion: Bien qu’il soit évident que le milieu de croissance APFECC peut supporter les cellules, des études supplémentaires doivent être menées pour réoptimiser le milieu de croissance. De même, bien que EWA puisse prendre en charge des cellules salivaires développées en 3D même après 20 jours, des études supplémentaires doivent être menées pour déterminer s'il existe des modifications phénotypiques dans la culture cellulaire et pour caractériser davantage l'échafaudage. Les deux produits doivent être testés avec des cellules salivaires primaires humaines afin de véritablement tester leur potentiel. Quoi qu'il en soit, les résultats présentés ici fournissent aux chercheurs de nouvelles idées et outils pour la culture de cellules salivaires en 2D et 3D, en fonction de leurs objectifs.

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Preface and Contributions of Authors

Hieu Michael Pham was responsible for culturing and expanding the NS-SV-AC cell line and huSMG-1 cell line; the development of the Egg White Alginate scaffold; the study direction and performance; and result analysis. Osama Elkashty and Owen Tao assisted with MTT assay and analysis in the media comparison study. Owen Tao also assisted with the degradation study and with changing cell culturing media. Li-Chieh Lin assisted with translating and writing the French version of the abstract. All other sections were written by Hieu Michael Pham and edited by Dr. Simon Tran. The project was funded by Canadian Institutes of Health Research (CIHR grant 119585), Natural Sciences and Engineering Research Council of Canada (NSERC grant 05247), and JWM Kim research fund.

1. Introduction

1.1 Salivary Gland and Saliva

Salivary glands are exocrine saliva-secreting tissues that reside in throughout the oral cavity. Human salivary glands consists of three major pairs of salivary glands which have unique characteristics; the sublingual gland is the smallest of the major glands, laying above the mylohyoid muscle but beneath the tongue, predominately secretes mucous-type saliva; the submandibular gland lies posterior to the sublingual gland and secretes a mixture of serous and mucous-type saliva; the parotid gland is the largest of the major glands, located near the ears wrapped around the mandibular ramus, and secretes mainly serous saliva [1].

These major glands are typically composed of two epithelial cell types: acinar cells and ductal cells. Acinar cells play a crucial role in salivary development. Depending on the gland, acinar cells can form mucous, serous, or mixed acini, which are responsible for saliva production and secretion. Similarly, ductal cells can form either intercalated, striated, or excretory ducts, which function to modify and transport the saliva. Specifically, ductal cells are able to make saliva hypotonic by selectively reabsorbing ions as it is being transported towards the oral cavity [2]. In addition, each type of ductal cells perform unique functions; for example, intercalated ductal cells contain granules that contain lysozyme and lactoferrin which are incorporated into saliva; striated ductal cells are unique in that they regulate the secretion and reabsorption of electrolytes; and excretory ductal cells are responsible for reabsorption of potassium and sodium, and the cumulative collection and secretion of saliva from multiple lobules [2]. An additional minor type of epithelial cells are myoepithelial cells which cover secretory lobules and primarily function to induce salivary flow upon stimulation by constricting the acini, causing saliva to flow from the acinar lumen towards the ducts [2]. Together, these epithelial cells form the tree-like foundational lobe structures in which saliva is produced and secreted through one common duct. Another cell type found in salivary glands is the mesenchymal cell. These cells play an important role in the growth and morphogenesis of epithelial cells and overall development of the gland [3]. Other accessory cells that contribute

to the salivary glands' overall function includes endothelial cells, fat cells, neural cells, and immune cells as these glands are vascularized and innervated [2, 4].

In addition, humans also have approximately 600-1000 minor salivary glands embedded within the mucosa of the buccal, labial, distal palatal, and lingual regions of the oral cavity and the pharynx [5]. Like major glands, minor salivary glands can also secrete serous, mucous, or mixed saliva, however, have a much less complex structure and morphology [5]. Unlike major salivary glands which collect and secrete saliva through a single duct, each minor salivary gland unit typically has its own singular duct in which they all simultaneously secrete through throughout the oral cavity which allows for effective lubrication [2].

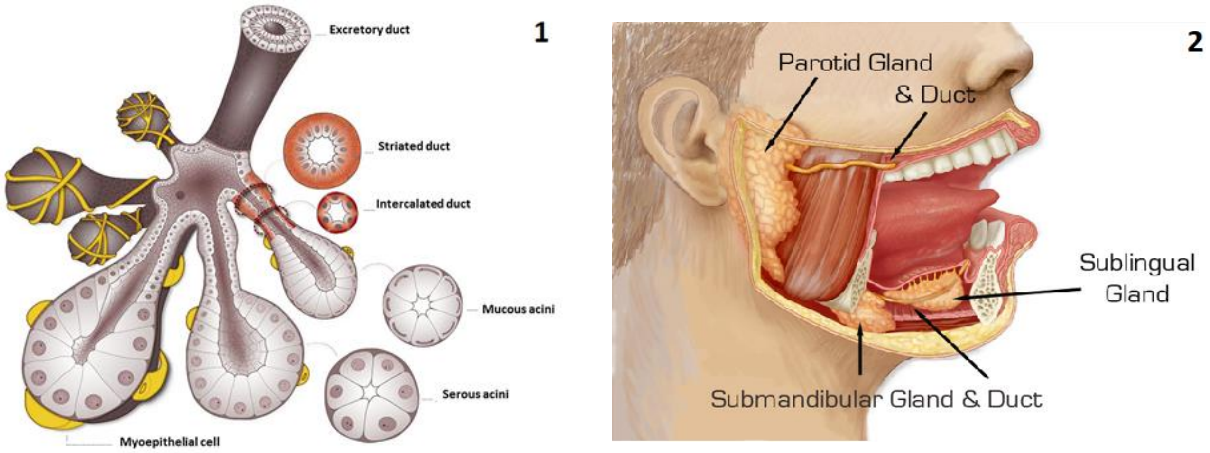


Figure 1. Secretory lobe anatomy. Illustration highlights the organization of various cells in the formation of mixed secretory lobes [2]. Image was taken from The Anatomical Record 2017; 300(7).

Figure 2. Locations of major salivary glands. Illustration displays the location in which each major salivary gland can be found [6]. Image was taken from Center for Advanced Parotid and Facial Nerve Surgery (n.d).

Together, these major and minor salivary glands allow the average individual to produce and secrete approximately 500-1500 mL of saliva each day, where 90% is produced by the major salivary glands (nearly 70% produced by the submandibular gland when unstimulated,

50% produced by the parotid gland when stimulated); the minor salivary gland contributes to the remaining 10% of total saliva secretions [7].

Saliva is a hypotonic solution, composed of more than 99% water, however contains a variety of components which enable saliva to perform its wide array of physiological functions including lubrication, digestion, maintenance of oral homeostasis, microbial protection, and tooth remineralization [8, 9]. Saliva may contain electrolytes such as sodium, potassium, calcium, magnesium, bicarbonate, and phosphate; proteins such as immunoglobulins, enzymes (α-amylase, salivary lingual lipase, lysozymes), and mucins; and nitrogenous compounds such as urea and ammonia [9]. In general, the major salivary glands contribute to the electrolyte concentration, while the minor salivary glands provide the proteins and blood-group substances. The concentration of each component typically varies depending on the flow rate and type of saliva [9]. Similarly, the pH of saliva is between 6-7, however can range between 5.3-7.8 depending on salivary flow rate. Tooth decay thrives in acidic environment (pH of 5-5.5), thus saliva plays a crucial protective role due to its innate buffering system, contributed from its bicarbonate, phosphate, and urea concentration. Therefore, major deviations from normal saliva composition or volume will impact oral health care [9].

1.2 Xerostomia

Xerostomia is a condition otherwise known as dry mouth syndrome in which patients have significantly reduced salivary flow rate (less than 0.1 mL/min) [7, 9]. The main causes of xerostomia are radiotherapy, Sjörgren's Syndrome (SS), and select prescribed drugs (mainly those with anticholinergic and sympathomimetic function) [7, 10]. Patients who have head and neck cancer (HNC) typically undergo radiotherapy and as a result have their salivary glands irradiated, meanwhile, those who develop SS—an autoimmune disease—experience destruction of their salivary cells via lymphocytic infiltration and attack [11]. Ultimately, both HNC and SS patients have dysfunctional/hypo-functional salivary glands [12]. Consequently, patients living with xerostomia tend to suffer collateral oral complications such as difficulty in speech, mastication, and swallowing; taste loss; and have increased susceptibility to dental

caries and oral infections and diseases [8]. Annually, there are approximately 500,000 new cases of HNC and roughly 400,000 new cases of SS worldwide [8, 13].

For these patients, the only methods of addressing xerostomia are palliative [10]. For example, frequent water sipping, using gel or spray saliva substitutes, and/or taking saliva-stimulating drugs such as pilocarpine hydrochloride or cevimeline hydrochloride are the primary means of lubricating the oral cavity [14]. Furthermore, SS patients do not have any curative treatment; the only preventative measure is to use immunosuppressive drugs, given the patient is experiencing severe systemic complications [15]. Likewise, while efforts are being made to improve radiation delivery techniques for HNC patients, approximately 40% of these patients still experience xerostomia post-irradiation therapy [16]. It is clear that treatment options are limited and insufficient despite the large population being affected, thus, there is an urgent need for the development of a curative treatment.

1.3 Salivary Tissue Engineering and Regenerative Medicine

There is a strong directional force in salivary gland research towards tissue engineering and regenerative medicine (TERM). The goal of TERM is to regenerate or grow a functional salivary gland which can serve multiple purposes. The artificial gland could be used for drug therapy testing, disease modeling and analysis, or even be transplanted, replacing the damaged salivary glands of HNC and SS patients. However, there are many obstacles that need to be considered in order to achieve success in TERM. Generally, the main fundamentals that need to be focused on are the graft cells, the scaffold hosting the cells, and the growth factors and nutrient nurturing the cells – optimizing these main aspects ensure the success of TERM. To date, there have been clinical success in skin, cartilage, bone, and bladder regeneration, however there is limited success with more complex tissues such as the pancreas, liver, heart, tooth, and salivary gland [17]. In order to grow a viable salivary gland that can serve the purposes previously mentioned, these glands need to resemble normal salivary glands, both phenotypically and functionally, and be autonomous if transplantation is being considered, to minimize risks of rejection.

To ensure normal physiological and functional development of the artificial salivary gland, several considerations should be addressed. For instances, the interactions between various cells should be considered as it can impact the morphogenesis, development, and differentiation of cells. Specifically, a study by Negawa and Mizuno (1981) suggested that salivary epithelial cell morphogenesis can be influenced by the presence of mesenchymal cells [18]. Other studies have shown that epithelial-mesenchymal interaction can also regulate tissue development and homeostasis [12]. Some studies have also shown that crosstalk between cells—that is, cell-cell contact and interaction—can influence morphogenesis and differentiation of submandibular salivary gland cells [19]. Another consideration is that the in vitro environment should resemble the specific microenvironment native to the respective cell; this includes temperature, oxygen and carbon dioxide saturation, pH level, stiffness/compliance of surrounding tissue, osmolality, and nutrition and growth factors [12, 20]. For example, in a study by Peters et al. (2014), they found that embryonic mouse submandibular salivary gland cells growing on a biomaterial with similar stiffness to embryonic salivary tissue (0.48 kPa) developed similar morphology to normal submandibular salivary gland cells. These cells also expressed key differentiation markers such as smooth muscle alpha-actin (SMA) and aquaporin 5 (AQP-5) in myoepithelial and pro-acinar cells respectively. However, when these cells were grown on a biomaterial scaffold with similar stiffness to cancerous salivary tissue (19.66 kPa), normal morphology and differentiation marker expression was interrupted [12]. In general, successful organogenesis is a rather complex task as the associated cells have a wide array of conditions and checkpoints that need to be met in order to develop as desired.

1.4 Current Scaffolds for 3D Cell Culture

In order to achieve organogenesis, cells need to be grown in a 3D environment in which cells can expand radially in all planes as opposed to laterally in a 2D environment. To date, many biomaterials have been developed and tested to meet this requirement in addition to the conditions mentioned in the previous section. These biomaterials can come in form of spheres, sandwich gels, porous scaffolds, or hydrogels [21]. Regardless, these biomaterials usually mimic the basement membrane native to salivary glands, which is typically composed of collagen IV,

laminin, nidogen, and proteoglycan perlecan/heparan sulfate proteoglycan 2 (HSPG2). The basement membrane is a special form of extracellular matrix that promotes cellular proliferation and differentiation; the ECM acts to direct cellular mechanisms by providing biophysical, biomechanical, and biochemical cues [22]. Currently there are a few notably hydrogels that are commonly used to culture cells, particularly salivary cells, in 3D. These hydrogels are typically composed of animal proteins to mimic a basement membrane, such as a collagen gel, fibrin gel, and Matrigel [22]. The latter example, Matrigel, is a hydrogel composed of extracellular proteins including collagen IV, laminin, fibronectin, entactin, and perlecan, secreted by Engelbreth-Holm-Swarm mouse sarcoma cell [10]. Matrigel is often considered the gold-standard scaffold for 3D salivary cell culture however as with every scaffold, there are advantages and disadvantages. For example, while many studies have successfully grown and studied 3D salivary cells using Matrigel, it is rather expensive relative to other scaffolds. Additionally, because it is composed of proteins from mouse sarcoma cells, there is very little clinical relevance as it cannot be used in humans.

While animal protein-based hydrogels support cellular differentiation, proliferation, migration and organization, and/or assembly, they generally lack tunability and reproducibility, and are potentially tumorigenic and/or immunogenic [22]. As a result, researchers have looked towards using other synthetic compounds to create a novel hybrid hydrogel. The most commonly used synthetic compound in salivary gland TERM are poly(ethylene glycol) (PEG) and hyaluronic acid (HA), though some researchers have also explored the use of other compounds including polyvinylidene fluoride (PVDF), poly(lactic-co-glycolic acid) (PLGA), chitosan, and rat or bovine collagen to name a few [22-25]. The variety in scaffold types accommodates the complexity required to adequately culture and direct normal cellular development. Depending on the goal, the different attributes unique to each scaffold type can result in different cellular phenotype. Additionally, these scaffolds may be chosen based on the criteria that researchers are seeking. Researchers may consider one scaffold over another depending on the degradation time, biocompatibility, affordability, accessibility, tunability of mechanochemical properties, and immunogenicity of the scaffold [22]. Evidently, no scaffold is the ideal scaffold as each

scaffold will have their own unique set of characteristics that make it more favourable in one situation over another – as a result, researchers continue to explore new scaffold alternatives to meet their research goals.

1.5 Egg White and Alginate

There have been extensive efforts in exploring novel scaffolds that could potentially improve cellular processes as previously highlighted. In recent years, there have been some evidence that the protein-dense component of the chicken egg, commonly known as the egg white, as a viable scaffold as it has been shown to support cellular attachment, differentiation, and survivability. While egg white is composed of a thick and thin albumen layer which is mainly composed of ovalbumin (< 50%), there are other proteins in the composition that resemble ECM proteins, acting as a substrate for cellular attachment [26, 27]; these ECM and ECM-like proteins may explain the results seen in several studies to date. For example, a study by Kaipparettu et al. (2008) reported that epithelial breast tumor cell lines grown on egg white have comparable phenotypes as those grown on Matrigel [26]. Similar observations have also been noted in human umbilical vein endothelial cells grown on egg white and Matrigel [27]. These studies highlight the feasibility of using egg white as an alternative biomaterial to Matrigel as it is able to produce similar results in two different cell types.

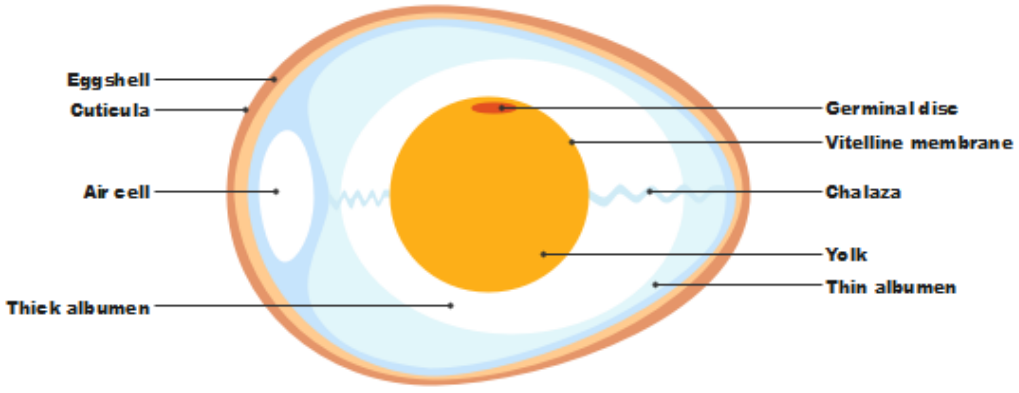


Figure 3. Unfertilized chicken egg anatomy. The component of interest is the egg white which is comprised of the thick and thin albumen [28]. This image was taken from EdrawSoft (n.d).

There are several rationales behind the use of egg white as an alternative to commercially available scaffolds. Firstly, it is a key component in the development of the chicken embryo into a baby chicken; the egg is capable of supporting a wide array of cell types and stages, suggesting its use in salivary gland cell culturing. Secondly, egg white is naturally transparent which enables the researcher to easily observe, monitor, and analyze cells grown on it. Additionally, egg white is naturally antibacterial and has immunomodulating activities as the gelatinous property of egg white acts as a physical barrier while the high ovalbumin concentration provides antibacterial properties [26]. Finally, unlike Matrigel and other conventional scaffolds, egg white is extremely inexpensive and highly accessible which may improve and/or accelerate TERM research [26]. However, like any biomaterial, there are also limitations to using egg white as a scaffold. A major limitation to using egg white is that the composition is difficult to determine. Due to its high ovalbumin and low minor protein concentrations, it is difficult to isolate and identify any minor proteins and its effect on cellular activity [29]. Additionally, while the viscosity of egg white can somewhat be tuned via temperature, temperature may also induce other undesired effects such as the loss of water and carbon dioxide, thinning of albumen, increase in pH, and changes in protein conformation [26, 30].

The poor tunable mechanical property of egg white can however be overcome by using egg white in conjunction with other materials such as alginate which has superior mechanical tunability. The stiffness, elasticity, compressibility, viscoelastic behaviour, degradation rate, and shape amongst other physical properties of alginate can easily be manipulated by altering the crosslinking type and duration, concentration of the crosslinking solution, ratios of mannuronate (M) and guluronate (G) subunits, the length of G blocks, and the concentration of alginate (typically between 1-3%) [31]. Modification of such physical features become crucial in cell cultures because cell phenotype and cellular activity is dependent on the stiffness/compliance of the ECM or scaffold [12]. By optimizing the methods used to create alginate, researchers can obtain the desired physical properties specific to the target cell type. However, the major issue with using alginate alone is that it is an inert biomaterial – that is it

cannot support cells on its own. Thus, by harnessing the highly tunable characteristics of alginate and the ECM-like properties of egg white, the Egg White Alginate (EWA) biomaterial could potentially be a very inexpensive yet effective novel biomaterial for culturing 3D cells, particularly salivary gland cells.

1.6 Animal Product-free Media

Cell culture media is the primary source of exogenous nutrients for any cell culture; thus it is necessary to dissect and understand the impact of the parts in the media. As previously mentioned, it is imperative to focus on growth factors and nutrient to succeed in TERM. Currently, when culturing human salivary gland cells, the most commonly used method is to use a basal medium such as Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS). FBS is a conglomeration of carbohydrates, proteins, fats, hormones, growth factors, vitamins and trace elements, and non-protein nitrogen products. Because it is a source of diverse nutrients, this growth supplement can virtually support any human and animal cells. FBS is able to provide cells with the adequate nutrients and stimulants to enhance cell growth and proliferation, promote differentiated functions, induce cell attachment and spreading, detoxify proteases and toxic molecules, and maintain adequate pH levels [32].

However, researchers may look to use a serum-free media for several reasons. First, there are many uncertainties due to the components of FBS not being well-defined. Therefore, studies performed with FBS introduces confounding effects or may result in outcomes that are unwanted and/or unexplained. Similarly, data collected from cells grown in FBS may lead to variability in results due to inconsistent concentrations of each components in different batches of FBS. Annually, over 1,000,000 bovine fetuses are sacrificed to collect and produce approximately 500,000 litres of FBS – it would be highly improbable for the numerous components of FBS to have similar concentrations [32]. Furthermore, these inconsistencies can differ drastically; Gstraunthaler et al. (2013) determined the concentration ranges for various compounds including cortisol, growth hormones, and progesterone which was found to have a range of 0.1-23 ng/mL, 18.7-51.6 ng/mL, and 3-360 pg/mL respectively [33]. Additionally,

because FBS contains a vast variety of components at various concentrations, it is inevitable that some cells may thrive more than others leading to overgrowth of specific cells such as fibroblasts over other cells such as epithelial cells [32]. Other reasons may be ethics-related, particularly revolving around the treatment and sacrifice of pregnant cows and their respective fetuses; the current methods can be seen as cruel as it may cause the animals to suffer. Furthermore as previously mentioned, over 1,000,000 fetuses are sacrificed on an annual basis thus raising questions in regard to sustainability [32].

Though animal products such as animal serums, bovine pituitary extract (BPE), chicken embryo extract, ocular fluids, bovine milk fractions, or bovine colostrum tend to be an excellent source for an all-encompassing nutrition kit, there are concerns regarding biocompatibility. Glands nurtured with FBS and/or other animal products will likely cause adverse immune reactions in humans thus impede the possibility of transplanting glands grown in vitro. Thus, in lieu of the end goal of TERM, translational research, and advancements towards clinical research, there is a need to establish an animal product-free media specific for culturing salivary glands. The loss of FBS and/or BPE supplementation means that researchers need to narrowly determine the key components and formulation that allow for cell survival specific to the cells of interest [34]. Additionally, any other supplementations need to be in slightly higher concentration to accommodate the loss of nutrients from excluding FBS and BPE.

Typically, these animal-derived supplements contain a wide array of supplements hence its potency. FBS contains components necessary for cell survival and proliferation, while BPE contains components that are mitogenic – that is, it promotes cell proliferation. The components that perform these functions need to be retained or compensated for when removing animal-derived components. Components such as insulin, transferrin, and hydrocortisone are examples of these critical components that are generally required by most cell types. However, specificity of other less crucial nutrients will vary depending on the cell type [34]. For example, specific cells require specific growth factors to thrive e.g. neurocytes require the addition of nerve growth factors (NGF), keratinocytes require epidermal growth

factors (EGF), and endothelial cells and fibroblasts require basic fibroblast growth factor (bFGF) [35]. These specificity ranges beyond growth factors and can include specificity in hormones, vitamins, trace elements, and attachment factors such as ECM/ECM-like substrates [34].

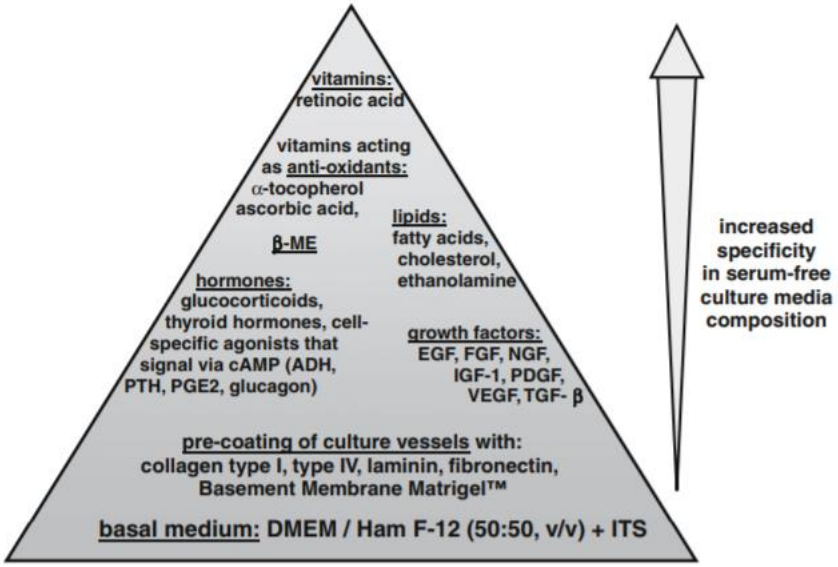


Figure 4. Specificity of the categories of supplementation. Basal media tend to accommodate a wider specificity of cells, while vitamins tend to be more cell-specific [34]. Image was taken from Toxicology in Vitro 2010; 24(4).

Once the necessary components are determined, there are additional aspects that need to be considered such as the impact of these compounds on the media's osmolality level. If the sums of the added compounds are too high—in which it exceeds the normal physiological range (approximately 260-320 mOsm/kg)—it creates an unfavorable environment thus may hinder cell proliferation [36, 37]. Another consideration to be made is the mode of delivery. Many of these nutritional components can be delivered to cells through its incorporation directly into the hydrogels such as Matrigel, however typically these components are added into the culture media as supplements [22]. There are many established basal media on the market that can be used such as minimum essential medium (MEM), MEM-alpha (MEM-α), Roswell Park Memorial Institute (RPMI) 1640, DMEM, and DMEM/Ham's F-12 (DMEM/F-12). The most commonly recommended basal media for a serum-free media for epithelial cells is DMEM/F-12 due to its

high nutrient content [34]. In general, there are many adjustments that need to be made when creating a media specific to cells of interest – this is even more complicated when excluded the use of universal animal-derived supplements. However, it is necessary to explore as these animal products cannot be used in humans as it will likely elicit an immunological reaction.

1.7 Rationale, Objectives, and Hypothesis

As a graduate student of McGill University, Faculty of Dentistry, the overarching goal of the research is to improve access to oral care, to improve current oral care interventions, and/or to address current oral diseases. As a student of Dr. Simon Tran's lab, this research aims to provide a novel method for those suffering with dysfunctional salivary glands through TERM. This thesis addresses the following issue by attempting to create a favourable environment for salivary gland cells grown in 3D that would resemble the normal human salivary gland; successfully grown artificial glands could then be used for future studies such as drug therapy testing, disease modeling, or far down the timeline – be transplanted into patients with missing or dysfunctional salivary glands. To create a favourable environment, this proposal takes a multi-directional approach by optimizing the scaffold and the culture media for these salivary gland cells. The scaffold can be thought of as the "home" of the cells that offers shelter and protection, while the media can be thought of as the "food" of the cells providing adequate nutrition. By optimizing the home and food supply of these cells, a stress-free and favourable environment can be created thus enable salivary glands to survive, proliferate, and differentiate.

Regarding the culture media, as the overall goal is to culture more biologically relevant salivary glands that can be used for human studies and purposes, this thesis explores the establishment of a novel animal product-free media specific for salivary gland cells. As previously mentioned, many popular media currently contain animal products even if it is "serum-free", meaning these cells will be exposed to animal proteins or antigens, thus if implemented in human studies, it may put the patient at risk of an immunological response. The need to establish an animal product-free media not only support sustainable and ethical

research (as no bovines and their fetuses—for example—would be sacrificed) but also facilitates the advancement of salivary gland TERM forward.

As previously highlighted, there is no perfect scaffold – only one that meets the goals of the researcher. The scaffold that will be used here is one that is composed of egg white and alginate, EWA. The intention of this scaffold is to provide researchers with a novel alternative to Matrigel that is more affordable and accessible. Currently, it costs approximately $19.09 (CAD) per millilitre to create a Matrigel scaffold for growing salivary gland cells compared to EWA which costs less than $0.08 per millilitre for the same purpose; i.e. EWA is 250X cheaper than Matrigel. Additionally, EWA is a much more sustainable alternative as it is created by combining the egg whites of regular chicken eggs with the extracts from the stems of *Laminaria hyperborean*, more commonly known as cuvie, a species of large brown kelp (algae) while Matrigel is created through the extracts of mouse sarcoma. Furthermore, the use of mouse sarcoma to culture salivary gland cells limits the possibility of using grown organoids in humans in future research due to its biocompatibility. Though EWA contains egg white and thus also raises questions about biocompatibility, it is at least more commonly exposed, is eaten, and is not derived from cancerous cells. Another benefit of using EWA over Matrigel lays in its physical properties. Because EWA is a hybrid hydrogel, containing alginate, it can easily be manipulated due to alginate's unique characteristics. The scaffold's stiffness and shape could be altered as desired, whereas Matrigel's tunability is relatively limited. As a result, those using EWA would have more control over how cells interact and develop which can aid in the goal of growing organoids. These advantages make EWA a worthy scaffold to explore, examine, and establish.

Several objectives have been outlined to address the overarching goal of improving salivary TERM research:

<u>Creating the Media</u>

a) Understand the components used in epithelial culture media.
b) Determine adequate replacements for animal product components in current culture media.

c) Create an animal product-free media that will sustain salivary cell cultures.

Creating the Scaffold

a) Establish the optimal protocol for the novel EWA scaffold.
b) Test physical and chemical parameters of EWA.
c) Demonstrate that EWA can sustain salivary cell cultures.

It is therefore hypothesized that the established animal product-free salivary cell media will be able to sustain salivary cell growth. Additionally, it is hypothesized that the Egg White Alginate scaffold will be able to grow and sustain salivary cell growth in 3D.

2. Materials and Method

2.1 Cell Culture

The cells used throughout the experiments include two cell lines: The normal salivary simian virus 40-immortalized acinar cell (NS-SV-AC) and the submandibular gland-human-1 (SMG-hu-1) cell lines. The NS-SV-AC cell line is a well-established cell line in the field of salivary gland research thus is a good candidate for this project. Contrastingly, the SMG-hu-1 cell line is novel and unstudied, however, it is used to ensure results with NS-SV-AC are replicable in other salivary cell lines. NS-SV-AC was donated by Professor Masayuki Azuma (Tokushima University Graduate Faculty of Dentistry, Japan) and Dr. Gulshan Sunavala-Dossabhoy (Louisiana State University Health Shreveport, USA) shipped to our lab, while SMG-hu-1 was established in the Tran laboratory by Li-Chieh Lin (McGill University, Faculty of Dentistry, Canada). These cells were cultured in an incubator at 37°C and 5% CO_2. The culture media used was typically Epi Max (Wisent Bio Products, 002-010-CL) supplemented with 1% 100X antibiotic-antimycotic (anti/anti) (Thermo Fisher, 15240062). The media was changed every 3 days, where the volume varied depending on the dish/plate type. A summary table can be found below (table 1).

Cells were passaged when dishes reached approximately 90% confluency. Cells were detached by first aspirating cell culture media, then rinsed 3 times for 15 seconds with 1X phosphate-buffered saline (PBS) (Thermo Fisher, 10010023), then a detachment solution of either 0.05% or 0.25% trypsin (GIBCO, 25200-056) diluted in ethlenediaminetetraacetic (EDTA) (Lonza, 17-711E) was used for NS-SV-AC and SMG-hu-1 respectively for 2 mins; the detachment solution volume varied depending on dish or culture plate type. During the 2 minutes, the dish/plate was repeatedly tapped on the sides and bottom to detach cells. The detachment solution was quenched by quickly adding 2 parts of DMEM (Thermo Fisher, 11995-065) + 10% FBS (Thermo Fisher, 12483-020) to arrest the digestive activity of trypsin. Cells were then further detached mechanically from the dish/plate by repeated pipetting of the detachment + quenching solution to flush any remaining cells. The solution was transferred to a 50 mL Falcon conical centrifuge tube which was then centrifuged at 300 G x 5 min at 4°C. The supernatant was carefully aspirated while the remaining cell pellet was resuspended and mixed in 1 mL of

culture media. The cells were then divided into new dishes/plates for continued culturing. Additional culture media was then added to the newly seeded dish.

To perform a cell count, prior to transferring the 1 mL of culture media + suspended cells, the 50mL tube containing the solution was pulse-vortexed and flicked several times to evenly suspend the cells. Then 10 µL of the solution was withdrawn and placed into a 0.6 mL Eppendorf microcentrifuge tube containing 90 µL of Trypan Blue (Thermo Fisher, 15250061). The microcentrifuge tube is then pulse-vortexed and flicked several times; 10 µL of the solution was transferred to a hematocytometer. Cells were counted on 5 blocks (4 corners + 1 central block) twice for a total of 10 sections. The total number of cells from the 10 blocks were then multiplied by 10,000 to obtain an approximate total number of cells in the 1 mL volume.

Table 1. Summary of solution volume used based on dish size or plate type

Dish Size	Growth Medium Volume	Detachment Solution Volume
100 mm	6 mL	3 mL
150 mm	14 mL	7 mL
Culture Plate Type		
6-well (2D)	1 mL/well	0.5 mL/well
6-well (3D)	2 mL/well	
24-well (3D)	300 mL/well	
96-well (2D)	100 mL/well	
96-well (3D)	200 mL/well	

* 2D Represents cells grown on plastic dish, while 3D represents cells grown on EWA or Matrigel.

2.2 Media Development – Animal Product-free Epithelial Cell Culture Growth Media

2.2.1 Literature Review

An extensive literature review was executed to explore the concentration values of various supplemental nutrients and the purpose it serves with respect to cellular processes. The

most common values (mode) found became the standard for that supplement. Some values were adjusted to be higher to compensate for the exclusion of FBS and BPE from the novel media. The search was performed using Pubmed and Google Scholar databases. Papers that involved the use of any keratinocytes or salivary gland cells grown in 3D were included. The search was limited to in vitro studies with no date restriction. The following search terms were used in various combinations:

"Salivary gland OR keratinocyte", "Salivary AND Supplement", "Salivary organoid OR salisphere OR 3D salivary cells", "Salivary AND Matrigel OR collagen OR ECM OR egg white OR chitosan", "3D Epithelial Cell Culture", "Serum-Free Media OR Animal-product Free Media", "FBS Composition OR BPE Composition", "Media Supplements", "Salivary AND X" (where X equals various supplements).

2.2.2 Media Preparation

Through the extensive literature review, it was decided that the basal media to be used in creating Animal Product-free Epithelial Cell Culture (APFECC) growth media would be

Section was modified for confidentiality purposes

The supplements were prepared by Wisent Bio Products' lab and was added directly into the media; they also corrected the medium's osmolality to 290 ± 20 mOsm/kg by adding 15-30 mL of double-distilled water (ddH_2O) into the media. Lastly, APFECC media was supplemented with 1% 100X anti/anti.

Other media used in the following study includes Epi Max and Keratinocyte Growth Medium – Gold (KGM-Gold) BulletKit (Lonza, 00192060). Both mediums were prepared with their respective supplements and 1% 100X anti/anti prior to use. MEM-α was also used as a

basal media for diluting MTT thus had no additional supplements. All media were stored in a 4°C refrigerator when not in-use.

2.2.3 MTT Assay

NS-SV-AC and SMG-hu-1 cells were plated in separate 96-well plates at a density of 5000 cells/well. Both cell lines were cultured in APFECC, Epi Max, or KGM-Gold media for 8 days; each media treatment was performed in triplicates. The cells were stored in an incubator at 37°C and 5% CO_2. MTT assays were performed on day 1, 2, 4, 6, and 8. A working solution of 0.5 mg/mL was created by mixing 1 part 10 mg/mL MTT (Sigma, M5655) dissolved in 1X PBS with 9 parts culture media (MEM-α) (Thermo Fisher, 12561049). To perform an MTT assay, cell culture media was carefully aspirated using a micropipette ensuring little to no contact with the bottom of the wells. Once the media is removed, 100 µL of MTT working solution was added into each well including empty wells for control. Plates were then covered with tin foil and placed into the incubator at 37°C and 5% CO_2 for 2 hours. The MTT solution was slowly aspirated with a micropipette ensuring minimal contact with the bottom of the wells. Once aspirated, 100 µL of dimethyl sulfoxide (DMSO) (Fisher Bioreagents, BP231-1) is added to each well including the control wells. The plates were the recovered with tin foil and placed on a titer plate shaker (Thermo Scientific, 4625) at 60 rpm for 5 min to dissolve precipitates. The plates were then placed onto a microplate reader (Bio-Tek Instruments, EL800) and read at an absorbance of 562 nm.

2.3 Scaffold Development – Egg White Alginate (EWA)

2.3.1 Scaffold Preparation

<u>Egg White Alginate</u>

First, the egg white (EW) is extracted from a chicken egg bought from a grocery store. The egg is sprayed with 70% ethanol to decontaminate under a biological safety cabinet (BSC). The egg is cracked on the tapered end by gentle tapping using the handle end of sterilized scissors. Once multiple cracks are formed, sterilized forceps were used to pry and remove the eggshell revealing the inner membrane with an area size of approximately 1 cm diameter. The

inner membrane is then slowly punctured with the forceps revealing the inner contents of the egg (figure 4h). The egg is turned over to pour the EW into a 50 mL conical centrifuge tube (figure 4c); forceps were used to pull the EW out. All other contents (chalaza, yolk, and watery content) were discarded; approximately 25 mL of EW was provided per egg. The tube is placed in an incubator at 58°C for 1 hour to sterilize (pasteurize).

Meanwhile, 2% by weight of Protanal LF 5/60 Alginate (FMC BioPolymer) was measured out on a balance (Adam Equipment Highland, HBC 123) under a BSC. The sodium alginate was added slowly (bit by bit at a time) into a 50 mL conical tube containing a solution of 1:3 Hank's Balanced Salt Solution (HBSS) (GIBCO, 14025076)/Media (either APFECC, or Epi Max). The solution was vigorously mixed by shaking and vortexing after each small addition of sodium alginate. The tube was then placed on a Speci-Mix Aliquot Mixer (Thermolyne, M71015) in a 37°C incubator for approximately 30 min to further mix and dissolve the alginate and any clumps formed (figure 4b). Calcium chloride ($CaCl_2$) (Fisher Scientific, C77-500)—the crosslinking solution—is created by dissolving 1% (by weight) calcium chloride into sterilized ddH_2O under a hood; the solution was shaken to mix well.

Finally, to create the EWA, 2 parts EW and 1-part sodium alginate was added into a separate 50 mL conical tube. The mixture was pipetted rigorously to mechanically break the EW and better mix the solution (figure 4d). Once homogenous, EWA mixture is centrifuged at 300 G x 1.5 min at 4°C to separate bubbles from the solution (figure 4e). A micropipette tip (with the tip cut off) was used to remove the top layer of bubble foam (figure 4a. A cut micropipette tip was then used to plate approximately 1 mL, 300 µL, and 100 µL of EWA into each well of a 6-well plate, 24-well plate, and 96 well plate respectively (figure 4f). Each well containing EWA was crosslinked with the $CaCl_2$ solution by slowly dripping 3 parts solution with a micropipette for every 1-part EWA or until the EWA solution was completely submerged (figure 4g). The EWA plate was then placed into a 37°C and 5% CO_2 incubator overnight to provide ample crosslinking time. The next day, the $CaCl_2$ solution was removed as much as possible. The EWA scaffold is then covered with culture media and stored in the incubator when not in use.

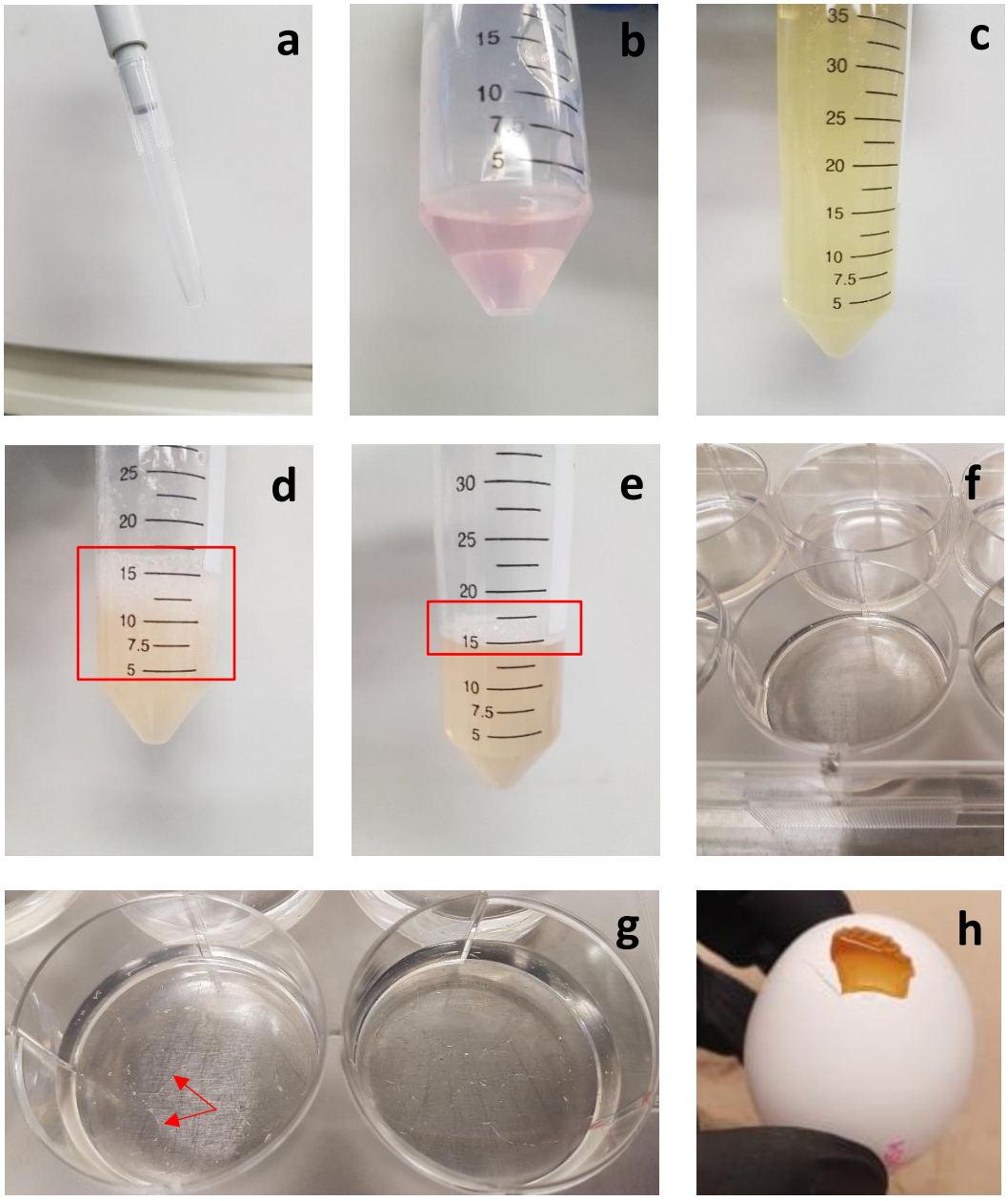


Figure 4 (a-g). Image guidelines for creating EWA. a) Image of a cut 1.25 mL micropipette tip. **b)** Image of 2% dissolved in Epi Max. **c)** Image of extracted EW. **d)** Image of EWA mixed together; lots of air bubbles throughout the solution. **e)** EWA after centrifugation; solution is homogenous and air bubbles form a foam layer at the top of the solution. **f)** Image of plated EWA prior to crosslinking. **g)** Image of plated EWA post-crosslinking (EWA gel submerged inside a $CaCl_2$ solution; red arrows highlight small crater structures due to crosslinking. **h)** Image of egg to highlight size of hole for EW extraction.

Matrigel

Matrigel (Corning, C356234) was thawed overnight in an ice bath in a 4°C refrigerator; frozen pipette tips were also placed in the -20°C refrigerator overnight. Under the BSC, the dish/plate was placed on an ice pack while Matrigel remained in the ice bath. Matrigel was transferred into a 15 mL conical centrifuge tube in an ice bath using a micropipette with the cold tips slightly cut (to widen the opening) and then was diluted with 5 parts of ice-cold culture media. The solution was mixed by repeatedly pipetting and pulse-vortexing. A cut micropipette tip was then used to plate approximately 1 mL, 300 µL, and 100 µL of Matrigel into each well of a 6-well plate, 24-well plate, and 96 well plate (sitting on an ice pack) respectively. The plate containing Matrigel was placed into a 37°C incubator to stiffen for 1 hour or until ready to plate cells.

2.3.2 Optimizing Alginate Percentage

EWA scaffolds were created as per above protocol using 1%, 2%, and 3% alginate solutions; EWA scaffolds were plated in 6 well plates, in triplicates (3 wells of each percentage). Approximately 50,000 NS-SV-AC cells were then seeded directly on to the scaffolds in each well using a micropipette. Cells were grown on the scaffold with the addition of Epi Max growth media and stored in an incubator at 37°C and 5% CO_2 for 5 days. Bright-field images were taken via light microscopy (Leica, DM IL) on day 5 at 50x and 200x magnifications. Images were taken at random for each triplicate. Once taken, the largest 5 spheroids in each image had its diameter measured and averaged out (total of 15 measurements per treatment) to obtain the average spheroid diameter size from each treatment.

2.3.3 Mechanical and Chemical Properties

Degradation Rate

EWA scaffolds were created as per above protocol in a 24-well plate in triplicates. Approximately 70,000 NS-SV-AC cells were then seeded directly on to the scaffolds in each well using a micropipette. The EWA and cells were submerged with Epi Max growth media which was changed every 3 days. The degradation rate was determined by measuring the solid weight

of the scaffold over 30 days. To weigh the scaffolds, the culture media was aspirated as much as possible using a micropipette. Then with sterilized tweezers, the scaffolds were picked up and placed onto sheets of Kimwipes for 10 sec to remove residual media. The scaffolds were then transferred to a pre-weighed plastic dishes and reweighed; the difference was then calculated.

pH Level

The 2% EWA was created using the above protocol however instead of combining a 2:1 ratio of EW/alginate, increments of 10% EW concentrations were prepared between 0-100%. These increments were also tested in 3 different conditions: prior to being crosslinked (no $CaCl_2$ solution added), after being crosslinked ($CaCl_2$ solution added), and after being crosslinked and media (Epi Max) is added. Each EW increment was created in triplicate and contained in separate 15 mL Falcon conical centrifuge tubes. The total EWA volume (prior to crosslinking) was 3 mL; as for the crosslinking trials, 3 mL of 1% $CaCl_2$ solution is added to each EWA tube (total of 6 mL) and allowed 12 hours to crosslink before measuring the pH; and with regard to the crosslinked EWA supplemented with culture media, the $CaCl_2$ solution was replaced with 3 mL of Epi Max after 12 hours of crosslinking time. The pH of each test was measured using a pH meter (Denver Instruments, UB-10). The pH probe was inserted, and the pH was record – this step was replicated 3 times. In between every measurement, the probe was rinsed with a pH 7.00 buffer solution (Thermo Fisher Orion, 910107). The pH of Matrigel with/without media was also measured for comparison; Matrigel was created with the above protocol.

2.3.4 Cell Viability

Live-Dead Cell Assay

NS-SV-AC and SMG-hu-1 cells were each seeded on 2% EWA created with the above protocol at a density of 150,000 cells/well in a 6-well plate in triplicates at each time point. Cells were cultured with Epi Max growth media which was refreshed every 3 days. Live-dead staining was performed on day 5, 10, and 20 using a Live and Dead Cell Assay kit (Abcam, ab115347). Live and dead stains were prepared by diluting stock solution of 4mM calcein acetoxymethyl (CalAM) and 2mM ethidium homodimer III (EthD-III) in 1X PBS to create a working solution of

2µM. The culture media was then removed as much as possible and 300 µL of each staining solution was distributed on top of the scaffold into each well. Plates were then covered with tin foil and incubated at room temperature for 1 hr. Both bright-light and fluorescent images were taken using the Leica DM IL microscope in the dark; images were taken at 50x magnification.

AlamarBlue Assay

NS-SV-AC and SMG-hu-1 cells were each seeded on 2% EWA created with the above protocol at a density of 30,000 cells/well in a 24-well plate in triplicates at each time point. Cells were cultured with Epi Max growth media which was refreshed every 3 days. Cell viability assays were performed on day 1, 2, 5, 7, and 10 using the AlamarBlue Cell Viability Reagent (Invitrogen, DAL1025); a working solution containing 10% AlamarBlue and 90% culture media (Epi Max) was made. Culture media was removed and replaced with 400 µL of the working solution for each well containing the scaffold and cells, as well as wells containing EWA only as controls. The plate was then covered with tin foil and left to incubate in an incubator at 37°C and 5% CO_2 for 6 hours. After the incubation period, 300 µL of the solution from each well was transferred to a 96-well plate as the physical presence of the EWA scaffold would interfere with the absorbance reading. The plates were then placed onto a microplate reader (Bio-Tek Instruments, EL800) and read at an absorbance of 562 nm and 595 nm.

2.3.5 Spheroid Formation

NS-SV-AC and SMG-hu-1 cells were cultured on EWA and Matrigel in 6-well plates, seeded at a density of 150,000 cells/well and performed in triplicates; control wells did not contain any cells. Cells were fed Epi Max growth media which was refreshed every 3 days, and were grown for 20 days in an incubator at 37°C and 5% CO_2. Bright-field images were taken via light microscopy (Leica, DM IL) on day 1, 2, 5, 10, 15, and 20 at 50x, 100x, and 200x magnifications. Images were taken at random for each triplicate. Once taken, the largest 5 spheroids in each image had its diameter measured and averaged out (total of 15 measurements per treatment) to obtain the average spheroid diameter size from each treatment.

2.3.6 Statistical Analysis

Statistical analysis was conducted using the Graph Pad Prism 6 package (GraphPad Software Inc., La Jolla, CA, USA). Various tests were used to analyze the data: A repeated measure (RM) one-way ANOVA, multiple comparison test with the Greenhouse-Geisser correction was used to analyze values between groups; a RM two-way ANOVA with Sidak's multiple comparisons test was used to compare values between groups over time. A p-value of <0.05 was considered as an indication of statistical significance.

3. Results

3.1 Media Development

3.1.1 Literature Search and APFECC Development

Two popular salivary growth media—Epi Max and KGM-Gold media—were used as controls to compare with the novel APFECC Media. In order to establish an adequate animal product-free media, an extensive literature review was performed to understand key components that must be incorporated into a growth media. The base media that was chosen for the establishment of the media was 50:50 DMEM/Ham's F12 basal media due to its high nutritional content. It was determined from the literature research that key supplementations that may pose benefits to salivary cell cultures were: recombinant human EGF, recombinant human bFGF, hydrocortisone, epinephrine, triiodothyronine, progesterone, recombinant human Insulin, recombinant human apo-transferrin, ethanolamine, selenium, retinoic acid, linoleic acid, linolenic acid, L-isoproterenol, adenine sulfate, uridine triphosphate, $CuSO_4$, gentamicin sulfate, and amphotericin B. However, while many of these incorporated supplements had extensive proof of benefits such as insulin, transferrin, ethanolamine, and selenium—commonly abbreviated as ITES—others had less support such as uridine triphosphate, linoleic acid, and linolenic acid. Despite the limited support, these supplementations were still incorporated as this was a pilot study and because there were little to no support against its use. Once the base media and its supplementation were decided, the formulation of the control media were obtained and cross-referenced and compared with (Table 2).

Table 2. Comparison of components found in cell culture media

Compound	APFECC Media	Epi Max Media	KGM-Gold Media
Inorganic Salts	**Concentration (µg/mL)**		
$CaCl_2$ (anhydrous)	[redacted]	--	--
$CaCl_2 \bullet 2H_2O$	[redacted]	222.00	Undisclosed
$CuSO_4$ (anhydrous)	[redacted]	--	--
$CuSO_4 \bullet 5H_2O$	[redacted]	Undisclosed	Undisclosed

$Fe(NO_3)_3 \bullet 9H_2O$	[redacted]		1.39	--
$FeSO_4 \bullet 7H_2O$			--	Undisclosed
H_2SeO_3			--	Undisclosed
KCl			186.25	Undisclosed
KH_2PO_4			68.05	--
$MgCl_2 \bullet 6H_2O$			--	Undisclosed
$MgSO_4$ (anhydrous)			180.57	--
$MnSO_4 \bullet H_2O$			--	Undisclosed
$MnSO_4 \bullet 5H_2O$			0.0001205	--
NaCl			7008.00	Undisclosed
$NaHCO_3$			2500.00	Undisclosed
$NaH_2PO_4 \bullet H_2O$			--	--
$Na_2HPO_4 \bullet 7H_2O$			--	Undisclosed
Na_2HPO_4 (anhydrous)			--	--
$Na_2SeO_3 \bullet 5H_2O$			Undisclosed	--
$Na_2SiO_3 \bullet 9H_2O$			Undisclosed	--
$(NH_4)_6Mo_7O_{24} \bullet 4H_2O$			Undisclosed	Undisclosed
NH_4VO_3			Undisclosed	Undisclosed
$NiCl_2 \bullet 6H_2O$			Undisclosed	Undisclosed
$SnCl_2 \bullet 2H_2O$			Undisclosed	Undisclosed
$ZnSO_4 \bullet 7H_2O$			Undisclosed	Undisclosed
Amino Acids			**Concentration (µg/mL)**	
L-Alanine			8.90	Undisclosed
L-Arginine			52.26	Undisclosed
L-Asparagine			132.10	Undisclosed
L-Aspartic Acid			13.31	Undisclosed
L-Cysteine			8.55	Undisclosed
L-Cystine ● 2HCl			--	--
L-Glutamic Acid			14.71	Undisclosed
L-Glutamine			292.00	Undisclosed
Glycine			7.50	Undisclosed
L-Histidine			15.52	Undisclosed
L-Isoleucine			13.12	Undisclosed
L-Leucine			39.36	Undisclosed
L-Lysine			29.24	Undisclosed
L-Methionine			4.48	Undisclosed
L-Phenylalanine			4.96	Undisclosed
L-Proline			5.76	Undisclosed
L-Serine			31.53	Undisclosed
L-Threonine			35.73	Undisclosed
L-Tryptophan			6.126	Undisclosed
L-Tyrosine			9.06	Undisclosed
L-Valine			35.16	Undisclosed
Vitamins and Others			**Concentration (µg/mL)**	
Adenine			0.1351	Undisclosed
Adenine Sulfate			--	--
Amphotericin B			Undisclosed	Undisclosed
Biotin			0.007329	Undisclosed
Bovine Pituitary Extract			Undisclosed	Undisclosed
D-Calcium Pantothenate (Vitamin B_5)			0.2383	Undisclosed
Choline Chloride			13.96	Undisclosed
Calcium Folinic Acid			0.006016	--
Cyanocobalamin (Vitamin B_{12})			0.013554	Undisclosed
Folic Acid			--	Undisclosed
D-Glucose			1441.6	Undisclosed
EGF, recomb. human			Undisclosed	Undisclosed
Epinephrine			--	Undisclosed
Ethanolamine			Undisclosed	Undisclosed
bFGF, recomb. human			--	--
Gentamycin			Undisclosed	Undisclosed
HEPES			7149.00	Undisclosed

Hydrocortisone	[redacted]		Undisclosed	Undisclosed
Hypoxanthine, Na			--	
i-Inositol			18.02	Undisclosed
Insulin, recomb. human			Undisclosed	Undisclosed
L-Isoproterenol			--	--
Linoleic Acid			--	--
Linolenic Acid			--	--
Lipoic Acid			0.002063	Undisclosed
Methyl Linoleate			--	--
Nicotinamide			6.1050	Undisclosed
Phenol Red, Na			1.242	Undisclosed
o-Phosphoethanolamine			Undisclosed	Undisclosed
Progesterone			--	--
Putrescine ● HCl			0.0001611	Undisclosed
Putrescine ● 2HCl			--	--
Pyridoxine ● HCl			0.02056	Undisclosed
Retinoic Acid			--	--
Riboflavin (Vitamin B_2)			0.11292	Undisclosed
Sodium Acetate ● $3H_2O$			--	Undisclosed
Sodium Pyruvate			110.00	Undisclosed
Thiamine ● HCl (Vitamin B_1)			0.3373	Undisclosed
Thymidine			0.07266	Undisclosed
Transferrin, recomb. human			Undisclosed	Undisclosed
Triiodothyronine			--	--
Uridine Triphosphate			--	--

* The placeholder "--" represents the absence of a compound in the media. All values are shown as concentrations in ug/mL. If the company kept the concentration of a component confidential, then "Undisclosed" was used as a placeholder.

Though Wisent provided a thorough formulation for their Epi-Max growth media, Lonza did not provide the concentrations to their KGM-Gold media; the exact component concentrations of KGM-Gold were not disclosed. However, it was determined that the current version of the base media was chronologically based on and modified from MCDB 153, to KBM-1, to KBM-2, and now KBM-Gold. The media was refined to improve its ability to support keratinocyte cultures at all densities, provide rapid cell growth and additional population doublings, and higher seeding efficiencies according to Lonza. Through this information it is possible to obtain a close estimation of the curtain concentrations found in the KGM-Gold Media.

In cross-referencing the media, several mineral and trace elements that were found in both Epi Max and KGM-Gold media were not supplemented in APFECC media such as $CuSO_4$ ● $5H_2O$, $(NH_4)_6Mo_7O_{24}$ ● $4H_2O$, NH_4VO_3, $NiCl_2$ ● $6H_2O$, and $SnCl_2$ ● $2H_2O$. It is unsure if the omittance of these elements will result in a drastic effect. The formulations of the pre-established Epi Max and KGM-Gold media were obtained post-MTT testing thus further studies need to be performed after making some modifications to the APFECC formulation.

3.1.2 MTT Assay

The results from the MTT assay indicate that there are no significant differences between any media at any timepoint in either cell line. All media were able to sustain the growth of salivary cell lines for the duration of the study. While there are no significant differences, it is worth noting the trend that Epi Max elicits more cell proliferation in both cell line at nearly every time point. In figure 5b and 5c, statistical significance was analyzed using a repeated measure (RM) one-way ANOVA, multiple comparison test with the Greenhouse-Geisser correction. The conclusion that can be drawn from this experiment is the use of APFECC is no better or worse than the use of Epi Max or KGM-Gold for culturing either cell lines.

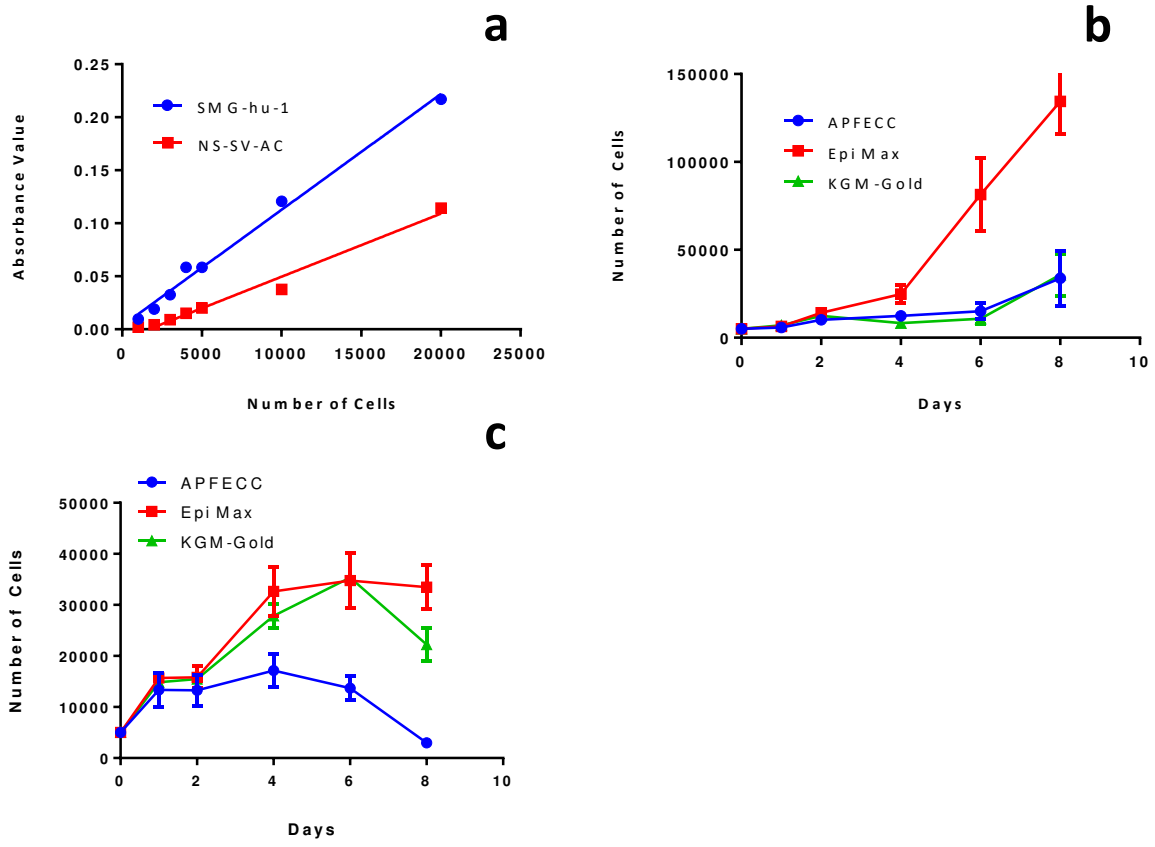


Figure 5 (a-c). MTT assay comparing media in culturing cell lines. a) This graph shows the correlation between absorbance value and total cell count. b) This graph shows SMG-hu-1 proliferation based on the different media. c) This graph shows NS-SV-AC proliferation based on the different media. The error bars shown represent the standard error (SE).

3.2 Scaffold Development

3.2.1 Optimizing Alginate Percentage

When observing the visual differences in spheroid formation across 1-3% EWA, it appeared that there was a significant difference in the 2% EWA compared to the 1% and 3% group as it had more cells and generally formed larger spheroids (figure 6). These differences could reflect the influence of scaffold stiffness on cell growth, migration, and survivability.

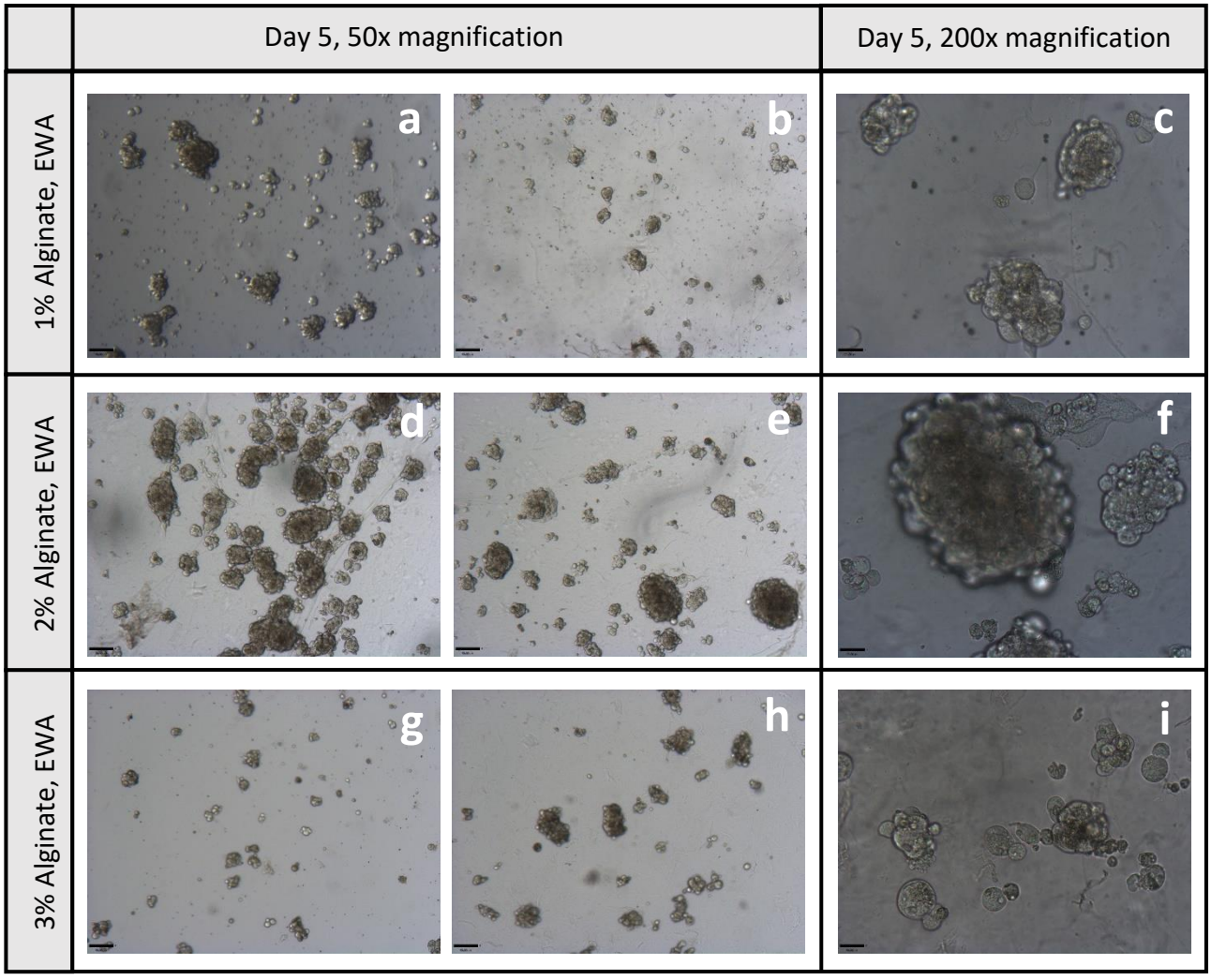


Figure 6 (a-i). Images of NS-SV-AC grown on EWA at 1, 2, or 3% alginate. Images a, b, d, e, g, and h were taken at 50x magnification while images c, f, and i were taken at 200x. The black bar on the bottom left of each image represents a scalebar where the bar length represents 90.00 µm and 22.00 µm respectively.

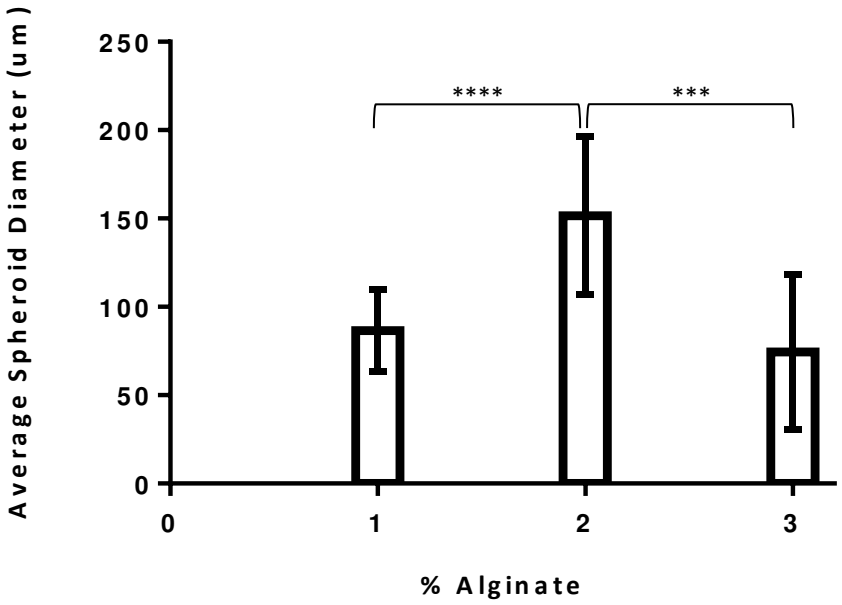


Figure 7. Comparison of spheroid sizes on different EWA alginate concentrations. The value of each bar shown was reported as a mean where N = 15 for each group. The error bars shown represent the SE of each group.

Through this short pilot experiment, it is apparent that regardless of the alginate percentage, NS-SV-AC cells can survive. However, it is visually and significantly evident that there are more NS-SV-AC cells in the 2% alginate EWA scaffold compared to the others. Additionally, the 2% alginate scaffold formed significantly larger clusters of cells compared to scaffolds composed of 1% ($p<0.0001$) or 3% ($p<0.001$) alginate. Statistical significance was analyzed using a RM one-way ANOVA, multiple comparison test with the Greenhouse-Geisser correction. It was also found that the absolute largest spheroid sizes (in diameter) were present in the 2% group compared to the 1% and 3% alginate group across all triplicates (data not shown). However, it is unclear if the difference in cell density and cluster size is due to the cell movement and clustering or due to proliferation. This data highlights the influence of scaffold stiffness on cellular function. It is inferred that the optimal scaffold stiffness for the NS-SV-AC cell line is equivalent to the stiffness of 2% EWA. Therefore, this pilot experiment suggests that an EWA scaffold composed of 2% alginate would be best for culturing NS-SV-AC in 3D.

3.2.2 Degradation Rate

Through measuring the degradation rate of the scaffold over 30 days, it was determined that there was an approximate loss of 0.002603 grams per day. In comparison to Matrigel, EWA degrades at a much slower rate. The data has not been shown here as the method of measurement is not feasible with Matrigel as it is far too soft and fragile. However, it is evident that Matrigel degrades quicker than EWA when culturing cells on each respective scaffold over 30 days. After 10 days, it is observable that Matrigel (plated at 1 mL/well in a 6-well plate) has become much softer than it was on day 1. Additionally, Matrigel further broke down into smaller islands after 10 days, as opposed to being one large scaffold shrinking in diameter over time as EWA would. Comparatively, EWA remains fully intact after 30 days, however, is smaller regarding overall volume, radius, and mass. This data suggests that the EWA scaffold is adequately robust and resistant to hydrolytic degradation as it remains fully intact with approximately 57% of its original weight after 30 days.

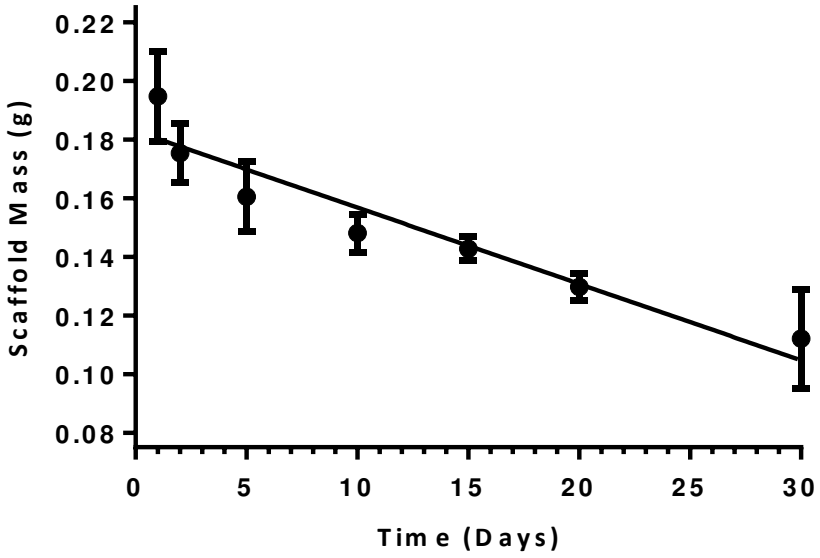


Figure 8. Degradation rate of EWA over time. The line of best fit is represented by the equation: y = -0.002603x + 0.1828. The error bars shown represent the SE.

3.2.3 pH Level

This experiment intended to characterize the optimal pH by adjusting the EW and alginate ratios. Figure 9 shows a linear relationship between EW percentage and pH level. As the EW percentage increases, the pH of the scaffold becomes more alkaline. Once the crosslinking solution—$CaCl_{2\,(aq)}$—is added, the pH of the scaffold appears to plateau. It is noteworthy that the EW percentage (post-crosslink) closest to the physiological pH (7.4) is at 60% EW; this is true when crosslinked with or without media. The pH of crosslinked 60% EWA with media is approximately 7.69 which is slightly more alkaline than physiological pH, however, is very similar to the pH of Matrigel with media (pH 7.65). While there are other EW percentages that are near physiological pH such as 80% and 90% EW, these percentages contain too high of an EW ratio which ultimately leads to the loss of the mechanical tunable properties of alginate, thus, an EWA scaffold containing roughly 60% EW would be more ideal. This data provides evidence for using a 2:1 EW/alginate ratio as the standard protocol for cell culture since Matrigel's pH level does not appear to be toxic to cells.

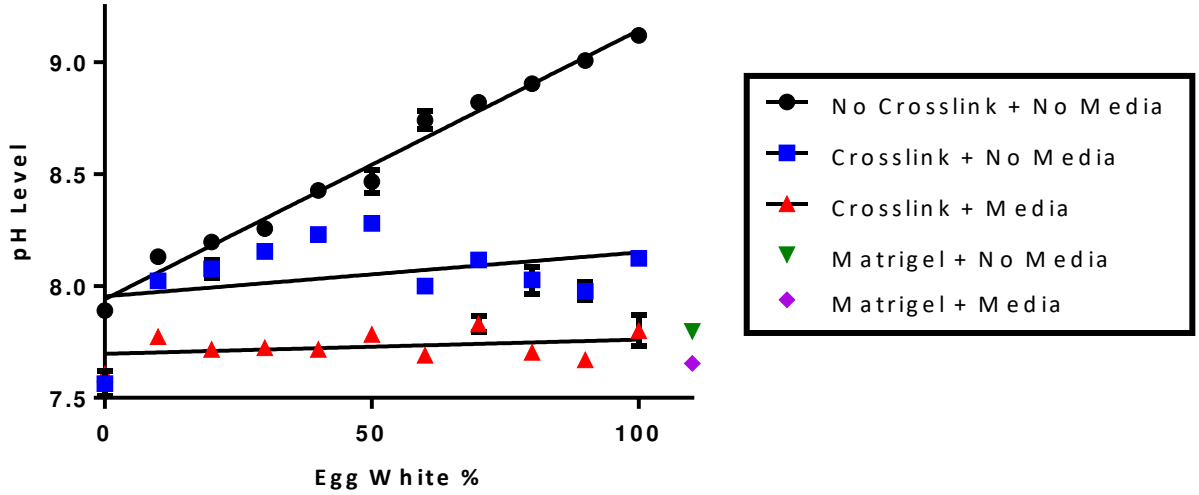
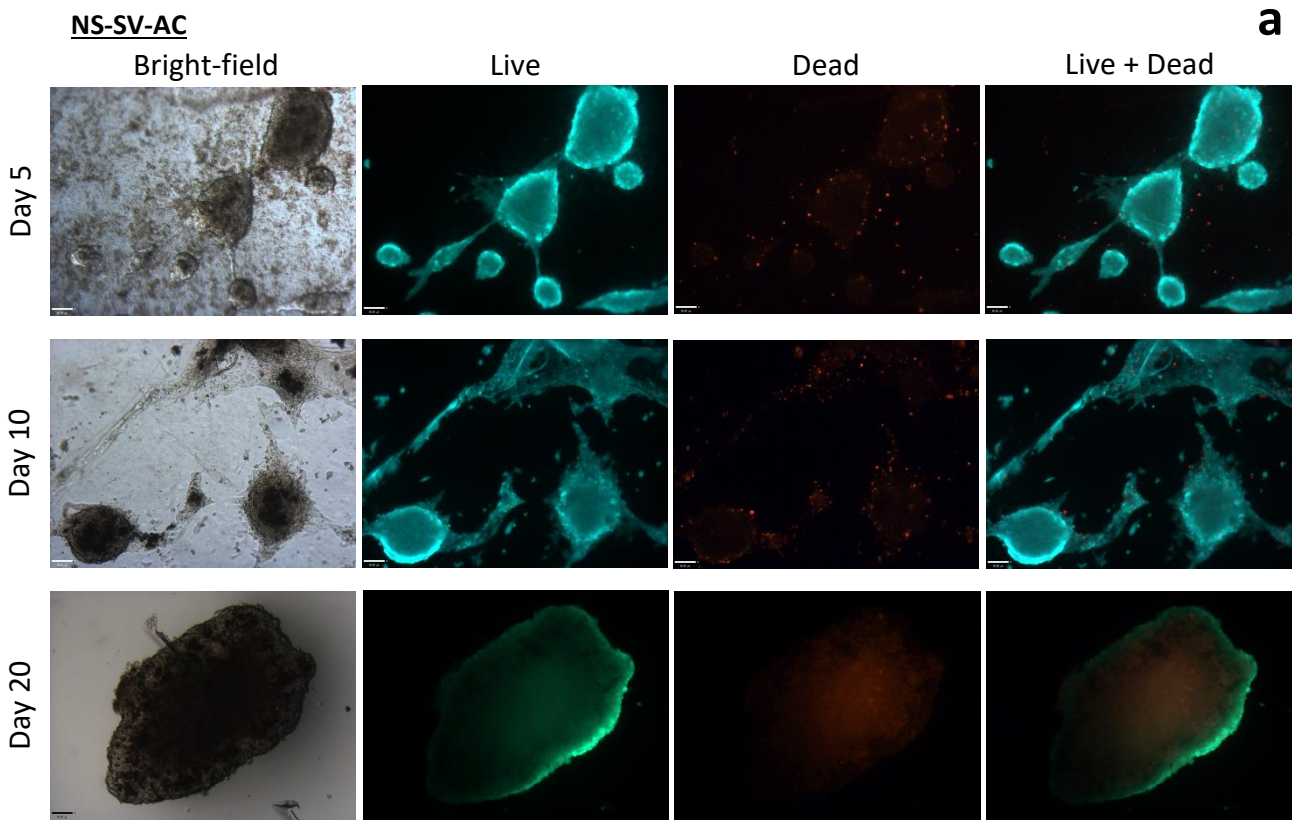


Figure 9. Correlation between pH level and EW percentage. These stages include prior to crosslinking (black), post-crosslinking (blue), and post-crosslinking with media added (red). The values of Matrigel with (purple) and without (green) media is also shown for reference. The error bars shown represent the SE.

3.2.4 Cell Viability

Live-Dead Staining:

Images of NS-SV-AC and SMG-hu-1 cells stained with CalAM and EthD-III were imaged to observe the feasibility of the EWA scaffold and its effect on cell viability. Through these images, it is evident that EWA was able to sustain cell growth after 20 days in both salivary cell lines. It is also evident that 3D spheroids are being formed over time, as the average size of each cluster of cell becomes larger. However, it is unclear if the size of these spheroids is due to cell proliferation or cell aggregation. These images highlight that many of these cells are live at day 5, 10, and 20, however, mainly on the surface of the spheroid. This suggests that EWA can serve as a feasible scaffold for cell culture and growth as cells exposed to such environment are surviving at all time points. However, once organoids of significant size form, necrotic cores begin to develop which may be due to hypoxia and malnourishment. Novel interventions that enable the delivery of oxygen and other essential nutrients to the core of these organoids need to be introduced to avoid hypoxia and thus necrosis.



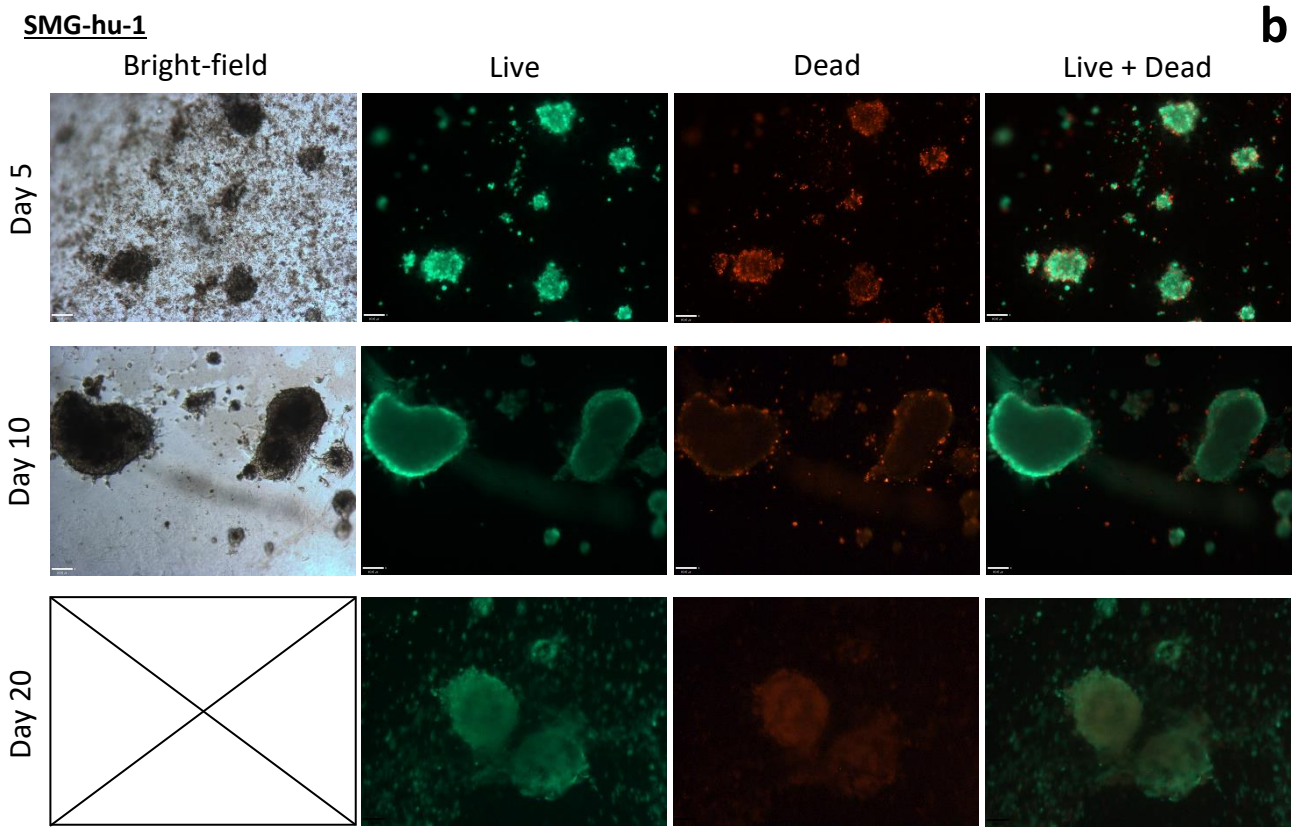


Figure 10 (a-b). Bright-light and Live-Dead images of cells on EWA. a) features NS-SV-AC (top 12 images) imaged with bright-light (first column), CalAM live stain (second column), EthD-III dead stain (third column), and a live-dead combination (fourth column), at 50x magnification on day 5, 10 and 20; the scalebar at the bottom left corner of each image represents 90 µm. b) displays the same type of images for SMG-hu-1 (bottom 11 images). The NS-SV-AC live stain images on day 5 and 10 had a blue tint due to being slightly photobleached. The SMG-hu-1 bright-field image on day 20 was lost.

AlamarBlue Assay:

An AlamarBlue assay was performed to obtain quantifiable evidence of cell viability on EWA. NS-SV-AC and SMG-hu-1 cells were seeded on 2% EWA and the cell viability assay was performed on day 1, 2, 5, 7, and 10; the percentage of AlamarBlue reduction was recorded, where a higher value indicates higher cell viability. The results reveal that neither cell lines result in an apparent linear trend; there is no significant growth over each time point in NS-SV-AC (blue) SMG-hu-1 (red) cell lines. The results suggest that EWA can support cell survivability

however there is no significant indication that cells are growing over the 10 days as there is no linear increase of AlamarBlue reduction percentage in either cell lines.

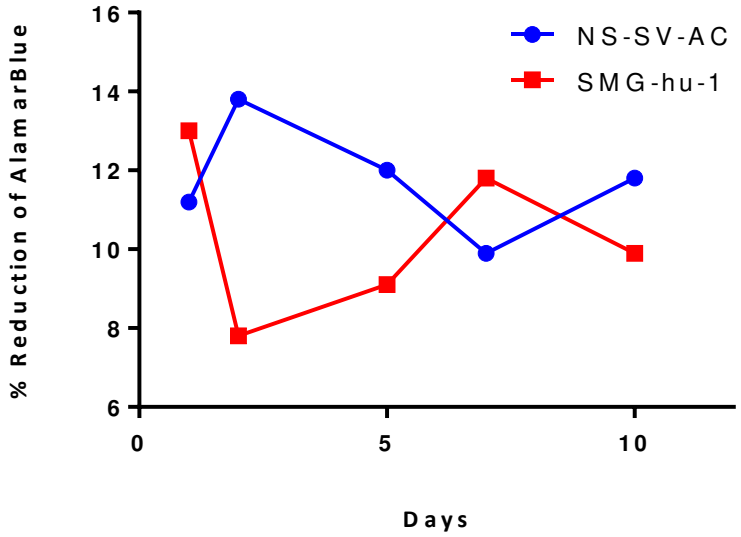


Figure 11. Measurement of AlamarBlue reduction percentage time. The blue (circle points) line represents the percentage of reduction of AlamarBlue for the NS-SV-AC cell line, while the red (square points) line represents the percentage of reduction of AlamarBlue for the SMG-hu-1 cell line.

3.2.5 Spheroid Formation

The intention of this experiment was to observe phenotypic differences of cells grown on different scaffolds. Regarding NS-SV-AC, there is clear evidence for formation of spheroid formation in both EWA (figure 12a) and Matrigel (figure 12b) treatments. It is likely that the formation of spheroids is due to cell clustering rather than cell proliferation. Evidently, from day 1 to 20, the number of single cells reduce drastically while the average spheroid size increases, thus suggesting that cells are migrating and clumping over time. Significance was found ($p<0.0001$) in both cell lines when considering the average spheroid size over 20 days thus providing evidence for spheroid formation. There are some differences between NS-SV-AC grown in EWA compared to Matrigel. For example, NS-SV-AC grown in EWA exhibit a larger average size with a dark centre as shown on day 10, 15, and 20. It is possible that the dark cores exhibited are necrotic cores as a result of the larger sphere size which shuns nutrients and

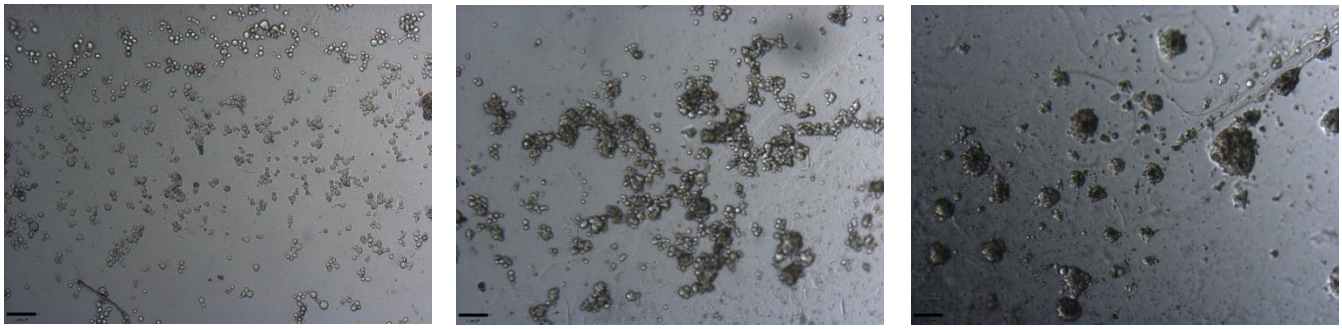
oxygen from inner cells. Additionally, on day 10, it is visible that there are 2D-like cells growing out laterally and connecting spheroids on the EWA scaffold. This lateral 2D movement could suggest that EWA allows for more cell migration compared to Matrigel, which may also explain the large spheroid sizes. It is possible that there is a compound that mimics an ECM structure that can act as a substrate in addition to EWA being a more rigid scaffold which enables cellular migration. The spheroids formed on Matrigel appear to be smaller than its counterpart grown on EWA. One complication with using Matrigel was that the suggested manufacturer's dilution of Matrigel for 3D salivary cell culture was too soft which resulted in the scaffold breaking quickly, resulting in cells sinking to the bottom of the plate. Conclusively, cells that fall to the bottom of the plate grow in 2D rather than forming 3D spheroids (images not shown here).

Regarding SMG-hu-1 cells, they do not appear to grow as well as NS-SV-AC in 3D. It is visually evident that in both EWA and Matrigel scaffolds, SMG-hu-1 cells appear to form smaller spheroids and have a lower cell density despite being plated at the same density as NS-SV-AC cells. This could be a result of cells undergoing apoptosis due to its inability to attach to the scaffold, however this is unclear and requires further investigations. When comparing SMG-hu-1 cells grown on EWA to that on Matrigel, the cells appear to be less compatible on EWA than Matrigel as the number of cells by day 20 in the EWA treatment is less than that of Matrigel despite having similar initial seeding numbers (data not shown here); this was not confirmed statistically. However, though there are more cells in Matrigel than EWA, spheroid sizes were larger in EWA than Matrigel. Visually, the cells between scaffolds look more similar than the NS-SV-AC cell line grown on EWA and Matrigel. The SMG-hu-1 cells grown on EWA and Matrigel appear to be similar in size though the cells on EWA are slightly darker in colour.

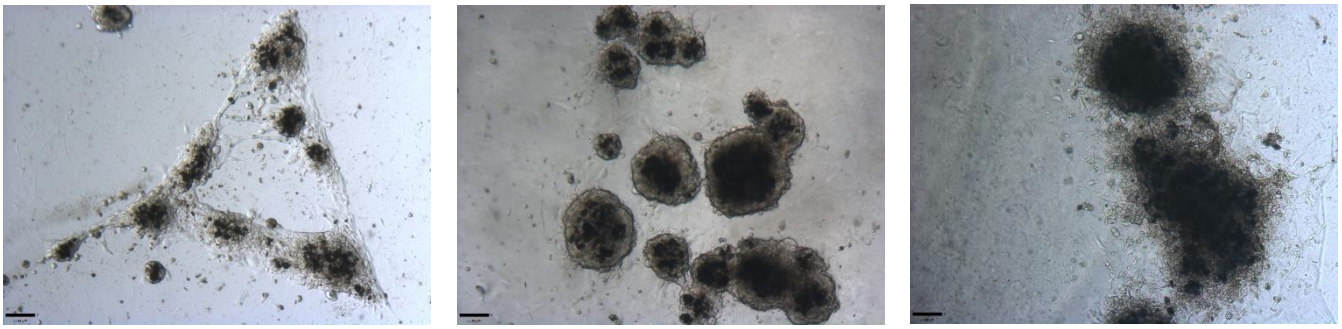
Using the images taken, the average spheroid size was also tracked over the timepoints and compare between the two scaffolds. The results indicated that while both scaffolds lead to larger spheroids over time, EWA allows for the formation of larger spheroid sizes compared to Matrigel – significance was found on day 20 in NS-SV-AC and day 10 in SMG-hu-1; though it is unconfirmed as to whether this indicates cell proliferation or not.

NS-SV-AC on EWA

a



Day 0 Day 1 Day 5

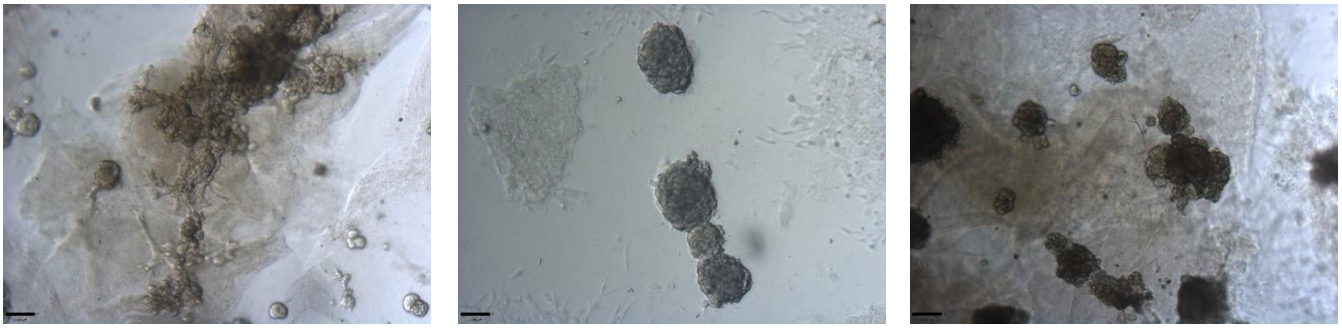


Day 10 Day 15 Day 20

NS-SV-AC on Matrigel

b

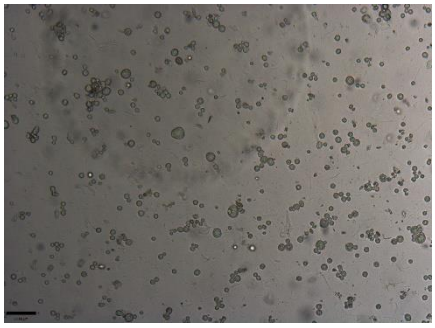
Day 0 Day 1 Day 5



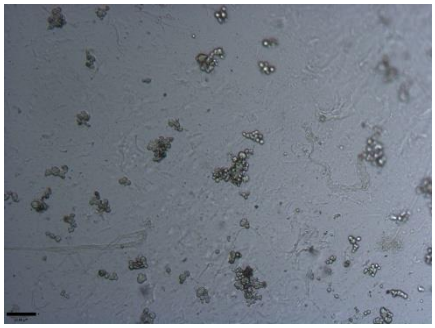
Day 10 Day 15 Day 20

SMG-hu-1 on EWA

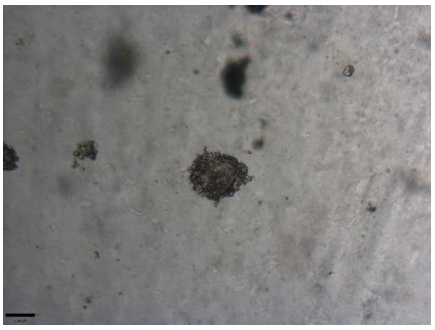
c



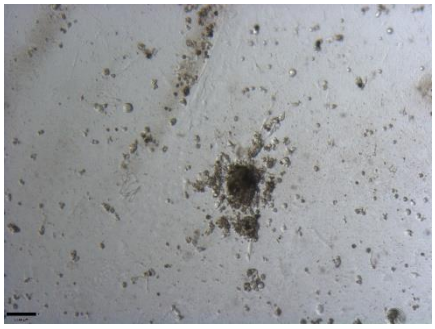
Day 0



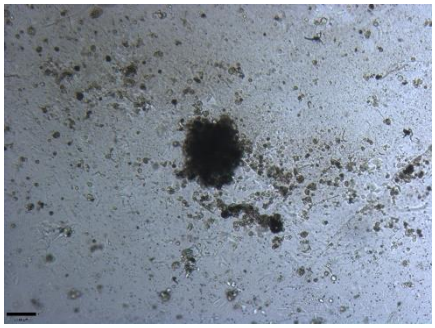
Day 1



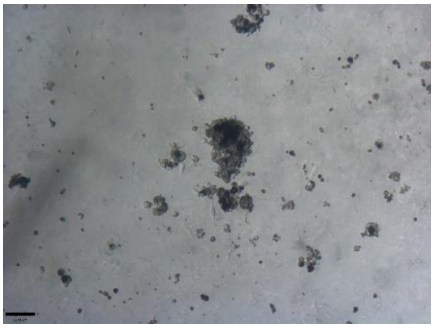
Day 5



Day 10



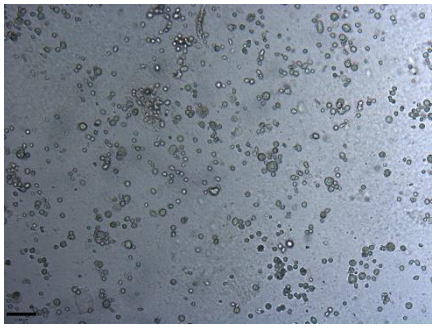
Day 15



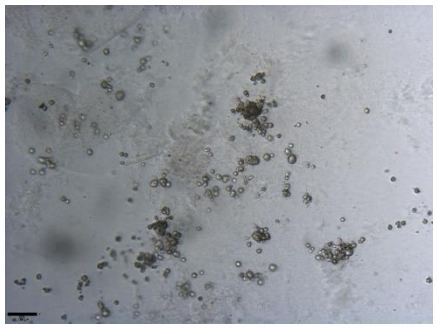
Day 20

SMG-hu-1 on Matrigel

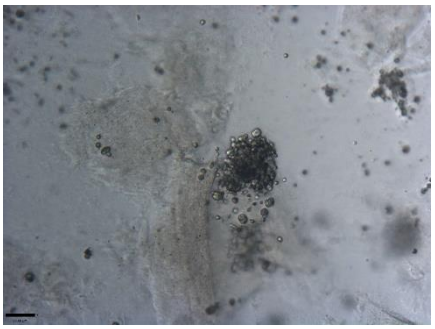
d



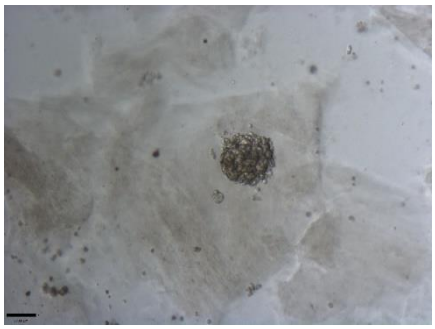
Day 0



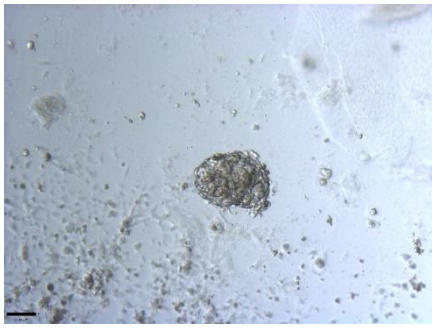
Day 1



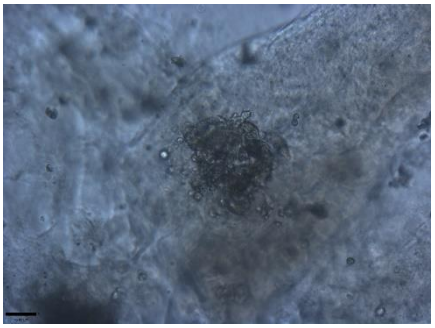
Day 5



Day 10



Day 15



Day 20

Figure 12 (a-d). Comparison of spheroid formation on EWA and Matrigel. a) NS-SV-AC cells grown on EWA; b) NS-SV-AC cells grown on Matrigel; c) SMG-hu-1 cells grown on EWA; d) SMG-hu-1 cells grown on Matrigel. All images were taken with a bright-light microscope at 50x magnification; images have different shades and lighting however it does not affect the cell features or scaling. Dark blemishes in figure b) and c) (day 5 and onwards) are due to Matrigel debris after being degraded over time. The black scalebars in the bottom left corner of every image represents 90 µm.

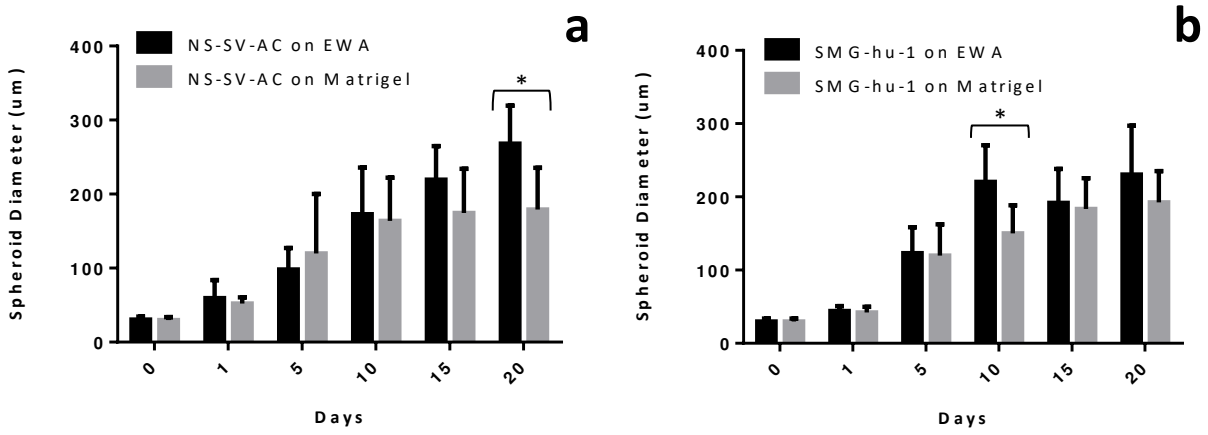


Figure 13. Comparison of average spheroid sizes between EWA or Matrigel. The value of each bar shown was reported as a mean where N = 15 for each group. The error bars shown represent the SE of each group.

When comparing the average spheroid sizes of NS-SV-AC and SMG-hu-1 cells grown on either EWA or Matrigel, significance was found on day 20 ($p<0.05$) in figure 13a. and day 10 ($p<0.05$) in figure 13b. when comparing average spheroid diameter between scaffolds. Differences in spheroid sizes between timepoints were extremely significant ($p<0.0001$). These statistical analyses were performed using a RM two-way ANOVA with Sidak's multiple comparisons test. Overall, this study served to provide a visual for the differences of cells grown on EWA and Matrigel. The study showed evidence for spheroid formation and the potential use of EWA as a novel scaffold for salivary cell culture. Additionally, it confirms that there are

morphological differences depending on the scaffold that contains the cells. However, further studies need to be performed to confirm if there are any differences in terms of individual cell morphology (opposed to spheroid morphology), protein expression, and cell function.

4. Discussion

4.1 Media Development

One scope of this research was to explore the use of a novel animal-product free media, APFECC. Through the results, it was determined that the APFECC growth media, was able to maintain cell proliferation despite the exclusion of BPE which is known to be mitogenic. The APFECC media was shown here to be equivalent to conventional media used for salivary cell cultures which contain BPE such as Epi Max and KGM-Gold growth media in both the SMG-hu-1 and NS-SV-AC cell line. While the differences in cell proliferation between the media treatments are insignificant, it is observable that Epi Max growth media allowed for the largest increase in cell proliferation compared to APFECC and KGM-Gold – this should be expected, as previously mentioned, the presence of BPE is mitogenic thus the media containing BPE should induce the most proliferation, given adequate supplementation of all other nutrients. Additionally, it was observable that cells did not appear to thrive in APFECC when seeded in a lower confluency that would otherwise be sustained by Epi Max or when cultured for a longer time period (data not shown here). It has been established that cell proliferation and survivability can be modulated by factors including initial cell seeding density, the growth period, and the production of endogenous self-addressed growth factors [38]. The combination between these factors—and possibly other factors such as lack of BPE—created a more stressful, less favourable environment for cells grown in APFECC media leading to apoptosis. As a result, it is likely that the media still needs to be refined and improved. However, because the media was established with such a diverse number of supplementations, it is difficult to determine which supplementation allowed for the results shown to occur. Thus, further studies that observe the individual benefits of each supplementation need to be explored to provide concrete evidence for each supplementation use.

There were some unusual trends found in the MTT test despite being performed in triplicates. For example, it is unclear as to why there is a downwards trend on day 4 of the NS-SV-AC test (figure 5c). Additionally, it is possible that there was an error in the SMG-hu-1 Epi

Max study (figure 5b (red)) as the number of cells increased at an abnormal rate beyond day 4. With the exclusion of the Epi Max treatment on SMG-hu-1 cells, other tests show a total number of cells that is below 50,000 cells at day 8, thus, for the Epi Max + SMG-hu-1 test to have approximately 120,000 cells is likely due to human error. It is possible that the cells were mis-seeded or the concentration of the MTT assay used was different. Regardless, this experiment should be revisited after improving the formulation for APFECC media. Some compounds found in both conventional media such as $CuSO_4 \bullet 5H_2O$, $(NH_4)_6Mo_7O_{24} \bullet 4H_2O$, NH_4VO_3, $NiCl_2 \bullet 6H_2O$, and $SnCl_2 \bullet 2H_2O$ were not incorporated into the APFECC media. Additionally, the concentration for these compounds were not disclosed, thus indicating the crucialness of their addition. Further literature research and experiments need to be performed to determine an appropriate concentration; however, it may be cell and/or cell line specific. With the goal of creating an animal product-free media for salivary gland cells, future studies should include the use of primary salivary cell as well rather than only salivary cell lines. However, historically, it is difficult without the use of FBS, thus the APFECC media will need to be improved further before testing with primary cells. Finally, a study should observe phenotypic differences in cells cultured in different media to ensure no abnormal behaviours will arise with the use of APFECC media.

Overall, through this study, it is clear that there is potential for the establishment and use of the APFECC growth media. As mentioned, the media needs to be closely studied to better understand the contributions of each components of the media on cell growth. However, this may be a challenging task as observing the individual effect of each supplementation on cells may not truly reflect the impact of the supplement, as often in biology, the whole may be greater or less than the sum of its part; some supplements may have synergistic effects, while some may have conflicting inhibitory effects. Additionally, the formula should consider different concentrations of the supplementation. For example, while many studies used a bFGF concentration of 5-10 ng/mL, they also had FBS and/or BPE which may contain an unknown concentration of bFGF as well. Thus, it may be beneficial to increase the concentrations of both EGF and bFGF to compensate for the lack of mitogens when refining the

formula. In a study by Kagami et al. (2000), they found that rat submandibular gland cells proliferated more when at least 100 ng/mL of bFGF was added to the culture media [39]. Increasing APFECC's bFGF concentration to 100 ng/mL may cause the cells to become more proliferative than the results in this study. Once these considerations are addressed, the new formulation should be tested on human primary salivary cells.

4.2 Scaffold Development

The results indicate that EWA is certainly feasible as a scaffold for salivary cell culture. Compared to Matrigel, it appears that EWA is more robust and enables both cell lines studied to form spheroids better. However, without future studies that identifies changes in gene and protein expression, and morphological changes, it is difficult to definitively state that EWA is better or worse than Matrigel. For example, with respect to the experiment observing the growth of NS-SV-AC grown in EWA or Matrigel, cells grown in EWA formed significantly larger spheroids and higher cell density, however this does not prove that it is better. While larger spheroids suggest more migration and/or proliferation, it could also suggest expression of oncogenic characteristics. Additionally, the size and morphology of the cells do not dictate whether the cells are functional or have anatomically correct cellular organization.

The NS-SV-AC cells grown in EWA were particularly interesting. Within these spheroids, a dark necrotic core could be seen under bright light (figure 12a) and was confirmed with a Live-Dead assay (figure 10a). The study confirmed that NS-SV-AC spheroids grown on EWA were significantly bigger than those grown on Matrigel, thus the core could be contributed to its size which shunts nutrients and oxygen from central cells. As a result, future studies should also look into the addition of a vasculature system whether it is one composed of an endothelial cell co-culture or an artificial inorganic system.

Regarding pH levels, Matrigel and EWA were not very different, 7.65 compared to 7.69 respectively. While the pH for both gels are similar, they are both slightly alkaline relative to

physiological pH. As shown by the pH study, EW is basic hence the alkalotic pH of EWA. It should be noted that some literature stated that egg white may have a pH as low as 7.6—closer to physiological pH—at the time of lay, however becomes more alkalotic over time (up to a pH level of 9.2). As a result, the time in which the egg is purchased and used to create EWA can influence the pH of EWA [40]. This time-sensitive pH level poses challenges because the time in which the typical chicken egg found in a supermarket is not disclosed and thus the current pH level cannot be predicted. Consequentially, due to pH being a function of time, EWA created with eggs at different times will have different pH levels thus may impact the consistency of future data and results. Perhaps this could be addressed by balancing the pH level of the EWA with a more acidic solvent while dissolving alginate (rather than culture media) to a standardized pH level such as physiological pH. However, a change in pH may alter the scaffold's degradation rate and mechanical properties, thus would require recharacterization of the scaffold [41].

Through these studies, other than spheroid formation, it is unknown if there are induced changes on cells grown on EWA compared to that grown in 2D or on Matrigel. Future studies should consider measuring cell proliferation rate, protein expression and location changes, functions, and the possibility of isolating and passaging the 3D cells. Because EWA is a novel hybrid scaffold, it posed some challenges that prevented these studies from occurring. Due to EWA being new, there was a lack of pre-established methods for isolating cells from the scaffold to perform certain studies such as immunofluorescent staining. Additionally, cell proliferation could not be measured using DNA isolation due to the scaffold containing trace DNA from the EW component. Because the volume of EWA is much greater compared to the volume occupied by the cells in the scaffold, any differences in the EW volume or DNA concentration would greatly skew the results of the DNA reading. Thus, new interventions or methods need to be established before one can consider measuring cell proliferation rate, protein expression and location changes, functions, and the possibility of isolating and passaging the 3D cells. A limitation in this study is that only salivary cell lines were used. With the end goal of TERM in mind, future directions should include the use of human primary

salivary cells to truly see the effectiveness of EWA as a scaffold for 3D salivary cultures. Similarly, the future studies should consider in vivo animal studies as Matrigel has been extensively studied in mice. This would provide further information of the scaffold's degradation rate, biocompatibility, and ability to culture cells.

5. Conclusion

This study has investigated the development and potential use of two novel products: an animal product-free media, APFECC growth media, and a more sustainable and affordable bio-scaffold, EWA. The purpose of this study was to recreate a growing environment for salivary cells with alternative goals. By establishing an animal product-free media, it facilitates the advancements in TERM and human interventions. By removing animal-based products from cell culture media, salivary organoids cultured in vitro can be used in human trials/studies because the cells would have not come in contact with antigens found in media containing FBS, BPE, or other animal-based supplements, thus would likely not elicit an immunogenic response. Creating EWA also facilitates this goal as it provides researchers with a more accessible, affordable, and sustainable scaffold for research. Though EWA contains an animal-based product, it is less invasive as it is not derived from cancer cells like Matrigel, however is likely still immunogenic; perhaps EWA will play a role in the search for an inexpensive and accessible animal product-free scaffold.

The goals set for establishing a novel culture media and scaffold were met in this study. In understanding the literature, an animal animal-product free media was created which proved to be statistically similar to culture media containing animal-based products. Though the results were statistically not different, it was visually apparent that the animal-based products cultured cells better than APFECC media. This suggests that some modifications to the media can be made to optimize cell growth and proliferation. Regarding the EWA scaffold, the scaffold was characterized and demonstrated that it has the potential to culture salivary cells in 3D. Although the scaffold could support 2 different salivary cell lines, it is unclear if proliferation is occurring. Many more studies need to be performed to further examine changes of cells in EWA compared to Matrigel and/or other scaffolds. Our hope is that the novel products established in this study mark advancements in TERM in support of patients suffering with xerostomia by providing researchers with new tools and insights to aid their work.

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7. Appendices

Appendix A: The Composition of Solutions

Table A1. Cell culture solutions

Epi Max Growth Media	**Amount**
Mammary Epithelial Basal Medium (Wisent Inc. 002-010-CL)	+ 500 mL
Epi Max 1 SH Supplement (Wisent Inc. 002-013-IL)	+ 5.00 mL
Epi Max 1 SA Supplement (Wisent Inc. 002-014-CL)	+ 5.00 mL
Epi Max 1 SG Supplement (Wisent Inc. 002-015-TL)	+ 0.50 mL
1% 100X Antibiotic-Antimycotic (Thermo Fisher, 15240062)	+ 5.00 mL
KGM-Gold Culture Media	**Amount**
KBM Gold Basal Medium (Lonza, 00192151)	+ 500 mL
KGM Gold SingleQuots Supplement pack (Lonza, 00192152):	
- Hydrocortisone	+ 0.50 mL
- Transferrin	+ 0.50 mL
- Epinephrine	+ 0.25 mL
- GA-1000	+ 0.50 mL
- BPE	+ 2.00 mL
- hEGF	+ 0.50 mL
- Insulin	+ 0.50 mL
1% 100X Antibiotic-Antimycotic (Thermo Fisher, 15240062)	+ 5.00 mL
APFECC Culture Media	**Amount**
DMEM/Ham's F-12 50/50 Mix (Wisent Bio Products, 319-081)	+ 500mL
Custom DMEM/F-12 Supplement 100X (Wisent Bio Products, 301-117)	+ 5.00 mL
1% 100X Antibiotic-Antimycotic (Thermo Fisher, 15240062)	+ 5.00 mL
Double-distilled water (osmolality correction)	+ 15-20 mL

Abbreviations: DMEM, Dulbecco's Modified Eagle Media; BPE, bovine pituitary extract; GA-1000, Gentamicin sulfate/Amphotericin B; hEGF, epidermal growth factor (human recombinant).

Table A2. Cell detaching-associated solutions

Trypsin	**Amount**
0.25% trypsin (GIBCO, 25200-056)	+ 100 mL
Diluted (0.05%) Trypsin	**Amount**
0.25% trypsin (GIBCO, 25200-056)	+ 5 mL
EDTA (Lonza, 17-711E)	+ 20 mL
Quenching Solution	**Amount**
DMEM (Thermo Fisher, 11995-065)	+ 500 mL
10% FBS (Thermo Fisher, 12483-020)	+ 50 mL
1% 100X Antibiotic-Antimycotic (Thermo Fisher, 15240062)	+ 5.00 mL

Abbreviations: EDTA, ethlenediaminetetraacetic; DMEM, Dulbecco's Modified Eagle Media; FBS, fetal bovine serum

Table A3. MTT assay solutions

Diluted MTT Reagent (working solution)	**Amount**
MTT (Sigma, M5655)	+ 100.00 mg
1X PBS (Thermo Fisher, 10010023)	+ 10 mL
MEM-α (Thermo Fisher, 12561049)	+ 90 mL

Abbreviations: MTT, thiazolyl blue tetrazolium bromide; PBS, phosphate-buffered saline; MEM-α, Minimum Essential Media

Table A4. AlamarBlue assay solutions

Diluted AlamarBlue Reagent (working solution)	**Amount**
AlamarBlue Cell Viability Reagent (Invitrogen, DAL1025)	+ 1 mL
Cell culture media	+ 9 mL

Table A5. Egg White Alginate scaffold solutions

2% Alginate	**Amount**
25% HBSS (GIBCO, 14025076)	+ 2.5 mL
75% cell culture media	+ 7.5 mL
2% (w/w) Protanal LF 5/60 Alginate (FMC BioPolymer)	+ 200.00 mg
EWA	**Amount**
33.33% Alginate	+ 10 mL
66.67% egg white	+ 20 mL
Crosslinking solution	**Amount**
1% (w/w) $CaCl_2$ (Fisher Scientific, C77-500)	+ 1.00 g
Double-distilled water	+ 99 mL

Abbreviation: HBSS, Hank's Balance Salt Solution.

Table A6. Matrigel solutions

Matrigel Scaffold	**Amount**
Matrigel Membrane Matrix (Corning, C356234)	+ 1 mL
Cell culture media	+ 5 mL

Table A7. Live-Dead assay solutions

Live Cell Stain Solution	**Amount**
CalAM (Abcam, ab115347)	+ 0.2 µL
1X PBS (Thermo Fisher, 10010023)	+ 399.8 µL
Dead Cell Stain Solution	**Amount**
EthD-III (Abcam, ab115347)	+ 0.4 µL
1X PBS (Thermo Fisher, 10010023)	+ 399.6 µL

Abbreviation: CalAM, calcein acetoxymethyl; PBS, phosphate-buffered saline; EthD-III, ethidium homodimer III.