

Original Article

Development of a Soy Protein-Based Composite Hydrogel as a Platform for Three-Dimensional Cell Culture of Human Salivary Glands

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Abstract

Background: Xerostomia, or dry mouth symptoms, affects billions of people worldwide and currently has no definitive cure. Tissue engineering with three-dimensional (3D) platforms has become increasingly popular for modelling salivary gland diseases. For decades, soybeans have been used in the pharmaceutical industry owing to their abundance of proteins, peptides, and amino acids, and recently, they have emerged as a source of sustainable biomaterials. **Methods:** A composite hydrogel from soy protein isolate (SPI), alginate (A), and gelatin (G) was developed by the hanging-drop technique and crosslinked with CaCl₂ for ionic gelation. The scaffold was characterized by scanning electron microscopy (SEM), fourier-transform infrared spectroscopy (FTIR), swelling and degradation rate, and mechanical tests. The *in vitro* biocompatibility of the scaffold on cellular proliferation, organoid formation, phenotype expression and functionality of two cell types: human submandibular gland cells (HuSMG) and primary salivary functional units (SFUs), was also assessed. **Results:** Soy-based hydrogels have a stiffness comparable to native salivary gland tissue (1–3 kPa) and controlled swelling and degradation rates after 3 weeks of incubation. Biological assessment indicated greater sustained cell viability (>90%) and better-organized spheroid/organoid structures in the soy-based hydrogels after 1 month of culture. Robust expression of key salivary gland markers, including zonula occludens-1 (ZO-1), aquaporin 5 (AQP5), cytokeratin 7 (CK7), α -amylase (AMY1) and α -smooth muscle actin (α SMA), was observed in both cell types cultured in the soy-based hydrogel. Additionally, the spheroids/organoids exhibited physiological responsiveness and functional maturation through agonist-induced stimulation, including ATP, Carbachol, and Isoprenaline. **Conclusions:** These findings validate the soy-based hydrogel as a biocompatible, sustainable and physiologically relevant 3D culture system for salivary tissue engineering and regeneration.

Keywords: Soy protein isolate, sustainable biomaterials, 3D culture, composite hydrogel, salivary gland regeneration, tissue engineering.

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Introduction

Salivary glands (SGs) are remarkably complex organs composed of interconnected ductal and secretory systems in the orofacial region [1]. There are three pairs of major SGs in humans: the parotid, submandibular and sublingual glands, along with numerous minor glands distributed throughout the mucosal lining of the oral cavity. These organs play an indispensable role in maintaining oral health by lubricating food, facilitating mastication, swallowing, and digestion [2]. When the glands are damaged by radiation therapy, viral infections, or autoimmune diseases [3,4], sequelae such as xerostomia, dental caries, halitosis, and oral infections can occur due to the organ's hypofunction

[5]. Unfortunately, the treatment of SG hypofunction is primarily palliative care, focusing on relieving symptoms and discomfort rather than addressing the underlying problem. Early work by Baum and colleagues [6–9] introduced three foundational concepts for SG regeneration: *in vitro* repair of irradiated glands, *in vivo* redesign of glands, and the development of artificial SGs. Their pioneering results have demonstrated the feasibility and potential of regenerative approaches in restoring SG functions.

In the field of tissue engineering, biomaterials, along with stem cells and growth factors, play a pivotal role in creating *in vitro* functional structures. Among these, hydrogels have emerged as one of the most widely applied

materials owing to their hydrophilic, cross-linked polymer networks [10]. Their popularity is largely due to their excellent biocompatibility, biodegradability, and high water content, which enable them to closely replicate the extracellular matrix (ECM) of natural tissue [11]. Hydrogels can be fabricated from both synthetic and natural sources, each offering distinct advantages and shortcomings. Recently, naturally derived hydrogels have gained considerable attention due to their biocompatibility, bioactivity and environmental sustainability. These biomaterials have been extensively developed for their potential to support cellular functions, integrate effectively with native tissues and facilitate organoid formation, offering a biologically relevant microenvironment that closely mimics the ECM, which supports cell adhesion, reorganization, and differentiation [11–13].

Currently, one of the commercially popular biomaterials in three-dimensional (3D) culture platforms is Matrigel®, a basement membrane extract from Engelbreth-Holm-Swarm mouse tumours. The material, rich in collagen IV, laminin, entactin, and other cell-binding domains, facilitates cell-to-cell and cell-to-matrix interactions, making it suitable for *in vitro* cell culture and organoid formation [14,15]. However, due to its origin from tumour cell lines, unpredictable effects *in vivo*, significant batch-to-batch variation, and unsustainability, Matrigel® has not been approved by the food and drug administration (FDA) and has rarely been applied in clinical trials [15].

As a result, there has been a growing interest in developing natural alternatives for 3D cell culture systems. Among these, soy-based materials have attracted significant attention for their nutritional value and therapeutic potential. High-quality sources of proteins from soybean, such as glycinin and β -conglycinin, provide essential amino acids that support cellular metabolism, proliferation and enzymatic activity [16], while soy-derived lipids and carbohydrates can contribute to maintaining cell membrane integrity [17]. Additionally, soybeans are rich in nutrients and bioactive compounds, including soy protein, lecithin, and isoflavones, which are widely recognized for their anti-inflammatory, wound-healing, cholesterol-lowering effects, and potential protective effects against cancer [18]. Numerous studies have successfully fabricated soy bioproducts, such as hydrogels, injectable pastes, thin films, and microcapsules for clinical applications [19–22]. Soy protein isolate (SPI), in particular, has demonstrated the capacity to form a stable hydrogel network held together by electrostatic interactions, disulfide bonds and hydrogen bonds, making it a promising scaffold material [22]. Several studies have recently explored the applications of soy components in SG regeneration. For example, a study by Ryo *et al.* (2014) [23] evaluated the effect of soy isoflavones on impaired SGs. In that study, patients with xerostomia were assigned to consume one 25 mg tablet of soy isoflavones daily. After 2 months, they reported signifi-

cant improvement in salivary secretion and reduction of oral discomfort, with no observed side effects such as sweating or polyuria [23]. These findings highlighted the antioxidant effects of soy-derived compounds and opened new possibilities for restoring SG functions. Recently, another group of scientists has also successfully combined SPI with sodium alginate to form an interpenetrating network (IPN) hydrogel for 3D cell culture systems. The IPN structure possessed enhanced compressive strength and elasticity, suggesting its potential for soft tissue engineering and muscle regeneration [24].

Building on this evidence, we propose a natural composite hydrogel, composed of SPI, alginate, and gelatin, as a 3D culture platform for salivary gland cells. In this system, SPI acts as the primary bioactive component. Rich in functional groups such as amines and carboxyl groups, SPI reinforces the hydrogel's structural integrity and offers antioxidant properties, which can promote cell viability by reducing oxidative stress in culture environments [25,26]. Gelatin, which contains Arg-Gly-Asp (RGD) sequences, provides adhesion sites for cell attachment through integrin receptors on the cell surface and helps to create a more uniform hydrogel network [27,28]. Alginate, through ionic crosslinking with divalent cations, forms a stable polymeric network with controlled porosity, stiffness and degradation rate [29]. To our knowledge, this is among the first studies to investigate soy-derived biomaterials for use in 3D cell culture platforms and regenerative applications of human salivary glands. By incorporating a plant-based component into a composite hydrogel system, we have demonstrated that the synergistic interaction of these three components has the potential to support long-term cell growth and functional maintenance of SG microtissues. Our soy-composite hydrogel has the potential to be a biocompatible, cost-effective, and sustainable 3D cell culture platform with future applications in drug screening and disease modelling in the field of SG tissue engineering and regeneration.

Materials and Methods

SPI with a protein content of more than 90% was obtained from Sigma-Aldrich (NISTRM8186). Sodium alginate powder (derived from brown algae, MW 100,000–200,000 g·mol⁻¹, guluronic acid content 65–70%) was purchased from Protanal LF 10/60 FT, FMC BioPolymer. Gelatin (bovine skin-derived) was purchased from Sigma-Aldrich, and calcium chloride dihydrate (CaCl₂·2H₂O) was purchased from VWR International, Belgium. All powders were sterilized by ultraviolet (UV) light overnight and stored at room temperature until further use. Hydrogel formulations were designed according to the formula S_xA_yG_z, with S for SPI, A for alginate, and G for gelatin, while their weight/volume percentages in PBS were represented by x, y, and z, respectively. As such, the formulation S2A2G7 would mean a concentration of SPI 2% (w/v), 2% Alginate (w/v), and 7% Gelatin (w/v). Accordingly, its com-

position was 0.2 g of SPI, 0.2 g of alginate, and 0.7 g of gelatin in 10 mL of PBS. This study had four groups of gels: A2G7 (the control gel without SPI), S0.5A2G7, S1A2G7 and S2A2G7. Phosphate-buffered saline (PBS) was prepared in our lab by dissolving 0.8g NaCl, 0.2g KCl, 1.44g Na₂HPO₄, and 0.24g KH₂PO₄ in 800 mL of distilled water, adjusting the pH to 7.45, then bringing the volume to 1 L, and sterilizing by autoclaving before further use. To prepare the gels, sterile powders were suspended in PBS by constant stirring at 37 °C and 500 rpm for 3 h using a magnetic stirrer. The mixtures were then subjected to an ultrasound treatment for 5 min, with a pulsed cycle of 8 s on and 2 s off, to ensure complete dispersion of the small particles. Tansaz *et al.* [19] reported that a 2% concentration of SPI represented the upper limit of its solubility under neutral pH conditions. At higher concentrations, maintaining a desirable pH becomes increasingly difficult in cell culture experiments. The selected concentrations were chosen to represent a systematic stepwise increase in SPI, with each concentration approximately doubling the previous one (from 0.5% to 1% and from 1% to 2%). This experimental design enabled us to efficiently evaluate the influence of SPI concentration on hydrogel properties while capturing concentration-dependent trends across a relatively broad range. Therefore, in this study, SPI concentrations of 0.5%, 1% and 2% were selected to ensure pH stability and provide optimal support for cell viability.

Preparation of hydrogel disks. After the preparation of the suspension, the mixtures were sterilized by filtration through a 0.22 µm syringe filter. Then, 30 µL of the resulting filtered solution from each group was hand-dropped onto a 100 mm dish. The droplets were left to rest at room temperature for 15 min to achieve partial surface gelation, to prevent surface irregularities and deformation. After 15 min, 0.1 M calcium chloride (CaCl₂) was added to the dish to initiate gelation through calcium-alginate ionic interactions. The samples were submerged in the CaCl₂ solution for 20 min. This gelation helped form stable 3D scaffolds. The hydrogel samples were then rinsed three times with PBS to remove residual calcium ions. Finally, they were transferred into new 100 mm dishes containing 5 mL of culture medium and incubated at 37 °C, 5% CO₂ for further experiments.

Fourier-transform infrared spectroscopy (FTIR). This experiment was performed to determine the chemical composition and molecular interactions within hydrogel samples. Measurements were performed by a Tensor 27 FTIR Spectrometer from Bruker Corporation, Massachusetts, USA, equipped with an attenuated total reflectance (ATR) accessory. Data were collected over a wavenumber range of 4000 to 400 cm⁻¹. Each spectrum was acquired by averaging 32 scans at a resolution of 4 cm⁻¹ to ensure a high signal-to-noise ratio and spectral clarity of the measurements. Hydrogel samples with varying concentrations of SPI were analyzed by FTIR to assess the incorporation of

soy-based components into the material's chemical structure.

Scanning electron microscopy (SEM). This experiment was performed to assess the surface morphology of the hydrogel scaffolds. Firstly, the gel disks were freeze-dried overnight to preserve their structural integrity and ensure complete removal of moisture. After that, they were sputter-coated with a 4 nm layer of platinum using a Leica EM ACE 600 high-vacuum coater (Leica Microsystems Inc., Wetzlar, Germany). This step was intended to enhance the conductivity of the samples for imaging. A scanning electron microscope (SEM; FlexSEM 1000II from Hitachi SU1000 Co., Ltd., Tokyo, Japan) was used to observe the surface topography of the dried hydrogels. SEM imaging was performed at a magnification of 100 × to capture the surface features of the hydrogels on day 1 and day 14. The images were collected from multiple randomly selected areas of three samples in each group. ImageJ software (version 1.54p, National Institutes of Health, Bethesda, MD, USA) was then used to quantify pore area; at least 100 pores per sample were analyzed. Results were reported as mean pore area (µm²) and mean ± standard deviation (SD).

Mechanical testing. This experiment was performed to evaluate the stiffness of hydrogel scaffolds. The test was conducted using the CellScale Microsquisher system and Squisherjoy software (CellScale Biomaterials Testing, Canada). Firstly, the hydrogel disks were cut into a cylindrical shape with a diameter of 1 mm and a height of 2 mm. Each sample was compressed by a microbeam at room temperature at a constant strain rate of 10 µm/s. During the experiment, force and displacement data were continuously recorded and converted into stress-strain curves. The compressive modulus was measured by calculating the slope of the linear region of the stress-strain curve (0–0.05 strain), which represented the most consistent range of elastic deformation in these materials.

Swelling and degradation rate. This experiment was conducted to understand the hydrogel's capacity to absorb water and its degradation behaviour over time in a specific application before breaking down. These behaviours influence the hydrogel's ability to simulate the natural ECM, providing suitable support during tissue formation and ensuring the scaffold's replacement at a suitable time. After hydrogel preparation as described above, 3–4 samples from each group were weighed on an analytical balance to assess the initial weight. The remaining samples were submerged in culture media (αMEM, 1% Anti-Anti) and incubated under standard cell culture conditions (a humidified environment at 37 °C with 5% CO₂) for 21 days. At predetermined time points (days 3, 7, 10, 14, 17, and 21), the samples from each group were carefully removed from the culture environments, gently blotted with filter paper to eliminate excess surface moisture, and weighed with the analytical balance. The percentages of swelling and degradation were calculated using the following equation: Eqn. (1).

$$\text{Weight change (\%)} = \frac{W_t - W_i}{W_i} \times 100\% \quad (1)$$

Where W_t is the sample weight at different time points, and W_i is the initial weight of the hydrogel samples. Positive percentages reflected swelling (weight change, %), while negative values indicated degradation or mass loss. Each experiment was performed at least at three independent time points ($n = 3$), with 3 samples per group ($n = 3$), and then the mean values were calculated and reported for each group.

3D cell culture in soy-based hydrogels. To optimize our 3D culture models, we first used human submandibular gland cells (HuSMG) in the experiments. This SG cell line was isolated using previously established protocols in our laboratory and was further immortalized for long-term experimental use [30,31]. The submandibular gland (SMG) was selected for the experiments as it is one of the major SGs in our body, producing the highest volume of unstimulated saliva. HuSMG cells consist predominantly of acinar cells, along with ductal and myoepithelial cells, making them a suitable candidate to evaluate the biocompatibility of our soy-based hydrogels. For further biological validation, we also compared our hydrogels with Matrigel®, a widely commercialized product for 3D culture. This comparison enabled us to critically assess the biocompatibility of our hydrogels for SG cell culture.

HuSMG cells were cultured in Epi Max I (mammary epithelial growth medium) supplemented with 1% Anti-Anti and 5% fetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% CO₂. Culture media were refreshed every three days. Cells at passages 3–6 were used for the experiments. Upon reaching 80–90% confluence, the cells were detached using 0.05% Trypsin-EDTA (Gibco), centrifuged, and resuspended. For each experimental group, 2×10^6 cells were mixed with 1 mL of hydrogel solution. Then the cell-embedded gel disks (30 μ L per drop) were carefully prepared as previously described. For Matrigel® preparation, Growth Factor Reduced Matrigel® Matrix (18.97 mg/mL, Millipore Sigma) was thawed overnight at 4 °C and kept on ice during the experiments to prevent premature gelation. A 12 mg/mL working solution of Matrigel® was prepared by mixing 0.63 mL of Matrigel® with 0.37 mL of cold medium. After collecting the cell pellets, we suspended 2×10^6 cells in the Matrigel® working solution and pipetted several times to homogenize. Then we carefully hand-dropped 30 μ L of the mixture into the center of each well in a pre-chilled 48-well plate. The plate was placed inside the 37 °C incubator for 20 min to allow gelation. After that, warm culture medium was gently added to each well. Cells were cultured in complete Epi Max I supplemented with 1% Anti-Anti and 5% FBS at 37 °C with 5% CO₂. The culture media were re-

freshed after three days to maintain optimal cell growth.

To further assess the biological performance of our most promising 3D hydrogel formulation, we cultured human salivary organoids, referred to as human salivary functional units (SFUs). These organoids were obtained from SG tissues of consenting patients undergoing craniofacial surgeries at the Maxillofacial Surgery Department, Victoria Royal Hospital. All procedures were conducted in compliance with ethical standards approved by the Research Ethics Board (IRB No.: A05-M62-05B). Donor SG tissues in this study were obtained from patients who had not experienced any prior radiotherapy or chemotherapy treatments to ensure the integrity of the samples. SFUs were isolated using enzymatic digestion protocols previously established by our lab [30] and subsequently embedded in the optimized soy-based hydrogel for further experiments.

Cell viability and proliferation assessment. Cellular proliferation was evaluated by the WST-8 assay. The Cell Counting Kit 8 (WST-8/CCK-8 Assay Kit AB228554, Abcam) was used at 7-day intervals over 28 days to measure the cellular metabolic activity, following the manufacturer's instructions. At each time point, four samples per group were transferred into a 96-well plate. Then, a mixture of 100 μ L of fresh culture medium and 10 μ L of WST-8 reagent was added to each well. The samples were incubated for 3 h at 37 °C. After incubation, the solution from each well was transferred to a new 96-well plate and measured using a microplate reader at a wavelength of 450 nm. The colorimetric readout of the assay was proportional to the number of living cells within the hydrogel.

Live/Dead staining. To assess cell viability, we stained the cells with the LIVE/DEAD® Viability/Cytotoxicity Kit for mammalian cells (Invitrogen, L3224). The staining solution was prepared by mixing 0.5 μ L of Calcein-AM (4 mM), 1 μ L of ethidium homodimer-I (2 mM), and 2 μ L of Hoechst 33342 (18 mM, TOCRIS Bioscience) into 1 mL of DPBS. The mixture was then gently pipetted and added to all the groups. The samples were incubated at 37 °C for 45 min for proper staining. After incubation, the staining solution was removed, and the samples were washed three times with PBS to eliminate excess dye. The Live/Dead staining assay was also performed at 7-day intervals for 28 days. Calcein-AM labelled live cells with green colour, while dead cells were stained red with ethidium homodimer-I, and nuclei were stained blue with Hoechst 33342. Fluorescent images were obtained using a confocal microscope (Zeiss LSM 980) with a Z-stack scan of 400–500 μ m thickness with 10 μ m intervals at 10 $\times$ magnification. The images were processed and analyzed using ImageJ software to determine cellular viability, proliferation and SG organoid sizes in 3D platforms.

Immunofluorescent staining. On the first day, the gel disks were collected and rinsed with NaCl/HEPES buffer (135 mM NaCl, 20 mM HEPES, pH 7.4). The hydrogels

were fixed with 4% paraformaldehyde (PFA) for 45 min and washed three times with NaCl/HEPES buffer for 15 min each. After that, 0.25% Triton-EDTA was used for permeabilization for 10 min. After three washes, samples were incubated overnight at 4 °C in a blocking solution (5% BSA in NaCl/HEPES buffer) to prevent non-specific binding. On the second day, the cells were stained overnight with different primary antibodies targeting specific salivary gland biomarkers such as aquaporin 5 (AQP5), epithelial cadherin (E-Cad), zonula occludens-1 (ZO-1), filamentous actin (F-actin), cytokeratin 7 (CK7), cytokeratin 5 (CK5), cytokeratin 14 (CK14), alpha-smooth muscle actin (α SMA), α -amylase (AMY1), and CD117 (c-kit). The next day, after triple washes, secondary antibodies diluted in 3% BSA were added and incubated overnight at 4 °C. Finally, the samples were washed three times with the same buffer and stained with DAPI (Invitrogen) for 20 min. To improve the imaging process, hydrogel disks were dissolved in a sodium citrate solution to retrieve the embedded cells. The cells were washed with PBS and placed on an 8-well chambered cover glass for microscope observation. A confocal microscope (Zeiss LSM 980) with a Z-stack scan of 400–500 μ m thickness, with 10 μ m intervals, at 20 $\times$ magnification was used to obtain the images, which were then processed and analyzed using ImageJ software.

Calcium assay. HuSMG spheroids and SFU organoids were retrieved from hydrogels and subsequently embedded in Matrigel within a chamber slide to ensure immobilization for observation. The secretory potential of the organoids was assessed using the Fluo-4 Calcium Imaging Kit (Fluo-4 AM, ThermoFisher, F10489). The staining solution was prepared according to the manufacturer's guidelines. After preparation, the Fluo-4 solution was added to each well containing the cells and incubated for 30 min at 37 °C. Then, the samples were removed from the incubator and incubated at room temperature for another 15 min at room temperature to allow proper staining. After that, Fluo-4 AM dye was removed, and the cells were washed again with DPBS (Ca^{2+} , Mg^{2+} free) before imaging. The cells were stimulated with three agonists, including Carbachol (100 μ M; muscarinic receptor agonist), ATP (100 μ M; purinergic receptor agonist), and Isoprenaline (100 μ M; β -adrenergic receptor agonist). DPBS without Ca^{2+} , Mg^{2+} served as a negative control. Time-lapse images were captured over 5 min with 2-s intervals using a confocal microscope (Zeiss LSM 900). Changes in fluorescence intensity were used to calculate the calcium responses in the experiment. All of the information, including catalog numbers, manufacturers, and locations of all instruments and reagents used in this study, is provided in **Supplementary Table 1**.

Data and Statistical Analysis. All samples were measured in triplicate ($n = 3$) and repeated independently at separate time points ($n = 3$). Data were reported as mean $\pm$ SD. Comparisons between two groups were conducted using an unpaired Student's *t*-test, while comparisons among three or

more groups were analyzed using one-way ANOVA, along with Tukey's Post Hoc or Bonferroni corrections. A *p*-value of less than 0.05 was considered statistically significant. Statistical analyses were performed, and graphs were generated using SPSS 22.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 10 software (GraphPad Software, San Diego, CA, USA).

Results

Physical and Chemical Characterization of Soy Composite Hydrogels

FTIR measurements were performed to confirm the chemical composition of the hydrogels by identifying the characteristic functional groups of each component. The structures of polypeptides and proteins are formed by amino acid units linked by different amide bonds, resulting in nine characteristic infrared absorption bands of protein, such as amide A, B, and amide I to VII [32,33]. As shown in Fig. 1a, the FTIR spectrum of gelatin exhibited a broad band around 3300 cm^{-1} that includes N–H and O–H stretching vibrations. A shoulder was observed at 3100 cm^{-1} , indicating C–H asymmetric stretching. The peak at 1600 cm^{-1} was characteristic of Amide I or C=O stretch, while the 1500 cm^{-1} peak represents Amide II, related to N–H bending or C–N stretching vibrations. Finally, the Amide III peak appeared at 1230 cm^{-1} , corresponding to complex vibrations of C–N stretching and N–H bending [33]. Regarding sodium alginate, a broad envelope around 3300 cm^{-1} was observed, corresponding to O–H stretching vibrations. Then, peaks at 1600 cm^{-1} and 1300 cm^{-1} indicated asymmetric and symmetric stretching vibrations of the carboxylate (COO^-) group, respectively. The peak around 990 cm^{-1} was associated with the C–O stretching vibration in the saccharide backbone [29,34].

In Fig. 1a, the FTIR spectrum of SPI showed a broad absorption band around 3200–3300 cm^{-1} , representing the O–H and N–H stretching vibrations. Additionally, the characteristic peak of SPI around 2900 cm^{-1} was attributed to aliphatic C–H stretching vibrations, specifically from $-\text{CH}_2-$ and $-\text{CH}_3$ groups. These vibrations originate mainly from the alkyl side chains of amino acids in the protein and may also include contributions from residual lipids that commonly remain after protein isolation. This was followed by the characteristic amide peaks: Amide I around 1600 cm^{-1} (C=O stretching), Amide II at 1500 cm^{-1} (N–H bending and C–N stretching), and Amide III at 1200–1300 cm^{-1} [19,35]. In Fig. 1b, the FTIR spectrum of A2G7 confirmed successful incorporation of alginate and gelatin into hydrogel formation. The broad peak at 3200–3600 cm^{-1} illustrated the overlapping O–H and N–H stretching vibrations. The region around 1650–1630 cm^{-1} showed the Amide I band of gelatin, which overlaps with the alginate COO^- asymmetric stretching. A small shoulder around 1540 cm^{-1} corresponds to Amide II of the gelatin component. The presence of alginate carboxylate bands at

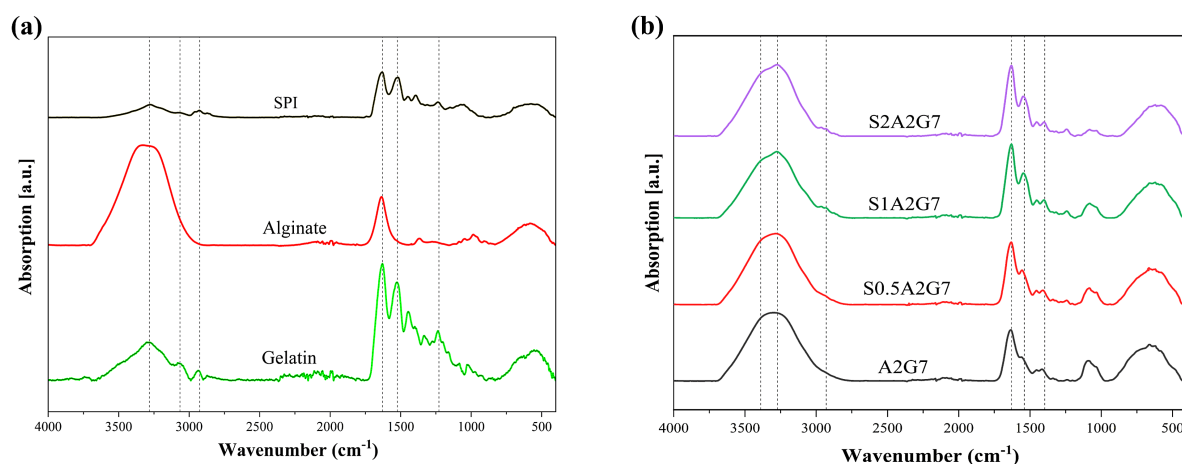


Fig. 1. FTIR spectra of hydrogel components. (a) FTIR spectra of gelatin, alginate, soy protein isolate (SPI) and soy-based hydrogel (S2A2G7). (b) FTIR spectra of different concentrations of soy-based hydrogels. The figure was created by OriginPro 2024 (OriginLab Corporation, Northampton, MA, USA).

1410–1440 cm⁻¹ and polysaccharide C–O–C vibrations at 1000–1100 cm⁻¹ further confirmed the hydrogel structure. With the incorporation of SPI, a peak around 3270 cm⁻¹ became more pronounced, which could correspond to the N–H stretching of peptide structures in the protein. The new 2900 cm⁻¹ band appearing in the soy-containing hydrogels corresponded to aliphatic C–H stretching from amino acid side chains. Additionally, the peak at approximately 1500 cm⁻¹ corresponding to Amide II showed enhanced intensity proportional to the concentration of soy protein. These peaks previously appeared in the pure SPI spectrum (Fig. 1a), with the concentration-dependent trend clearly mirroring the soy-composite hydrogel. These spectral changes suggested the physical entrapment of soy protein within the hydrogel network. The S2A2G7 group presented all the major absorption bands of soy protein, alginate and gelatin, confirming successful integration of all three components within the hydrogel matrix.

The SEM images in Fig. 2a showed the pores in the scaffolds and their changes in size after 14 days of incubation. As the SPI concentration increased, the scaffold structure became more porous, with a trend towards larger pores ($p < 0.05$). SEM images of pore morphology in different soy-hydrogel groups on day 1 were observed at magnifications of 100× and 500× in **Supplementary Fig. 1**. Pore size did not change after 14 days in culture media for any of the samples except for S2A2G7, which showed a significant decrease in pore size ($p < 0.05$) (Fig. 2b). This may be due to partial degradation of the hydrogel matrix and potentially the release of its components into the surrounding environment, causing the internal structure of the hydrogel to collapse, resulting in the shrinking of the pores

and a denser network of the matrix. Therefore, to clarify which components of the hydrogels were released into the surrounding environment over time, we performed the FTIR analysis on the incubation media collected on Day 1 and Day 21 (**Supplementary Fig. 4**). The media samples showed peaks around 3306 cm⁻¹ and 1640 cm⁻¹ corresponding to the O–H stretching and H–O–H bending vibrations of water, respectively. Specifically, the peak at 1023 cm⁻¹, indicating the C–O–C and C–O stretching vibrations associated with the polysaccharide backbone of the hydrogels, showed a significant increase in the S2A2G7 group on Day 21 compared to Day 1. This increase in intensity demonstrated a progressive release of polysaccharide components from soy-based hydrogels after long-term incubation. In contrast, the control group (A2G7) showed only a modest increase in intensity for this peak between the first and last day of incubation. This difference could be attributed to the incorporation of SPI, which altered intermolecular interactions, weakened the network structure, and facilitated more polysaccharide particles to be released into the environment after extended incubation time. This finding suggests that the incorporation of SPI may compromise the structural integrity and long-term crosslinking stability of alginate within the composite hydrogel. Consequently, the alginate network is increasingly susceptible to ionic de-crosslinking and subsequent diffusion into the culture medium after long-term incubation.

As shown in Fig. 2c, all hydrogels exhibited noticeable swelling after 5 days of incubation in culture media (αMEM, 1% Anti-Anti). The control group (A2G7) absorbed the fluid and swelled the most ($p < 0.05$) at the early stage, whereas the soy-based groups exhibited lower

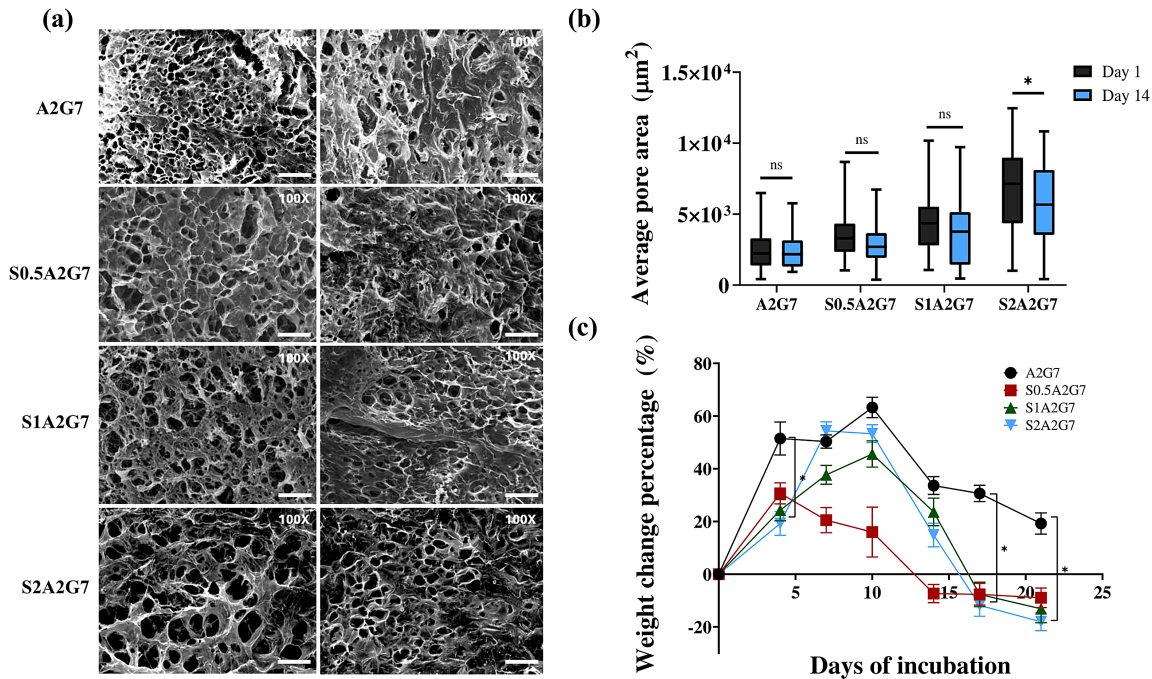


Fig. 2. Pore size measurement and weight change of soy-composite hydrogel. (a) SEM of soy-composite hydrogels on Day 1 (left column) and Day 14 (right column). Magnification/Scale bar: 100 $\times$ /100 μm . (b) Pore size comparison on Day 1 and Day 14 among the groups. (c) Swelling and degradation rate of hydrogels with different concentrations of SPI. Unpaired two-tailed Student's *t*-test was conducted for data analysis. Data are presented as mean $\pm$ SD, $n \geq 5$, n.s., not significant; *: $p < 0.05$. The figure was created by GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA).

swelling capacity and reached their maximum expansion at a later stage. By day 10, all groups had reached their peak swelling, and they started to degrade. Toward the end of the incubation period, the degradation rate became more pronounced. The control group (A2G7) exhibited the least degradation, while the SPI-containing hydrogels degraded faster and lost more weight. Especially, the S2A2G7 group demonstrated the most substantial weight loss (Fig. 2c), losing approximately 20% of its initial weight at the end of the experiment. The results confirmed our earlier conclusion that as SPI concentration in the hydrogel increased, the samples became more susceptible to degradation.

Soy-Based Hydrogels Exhibited Mechanical Properties Comparable to Human Salivary Tissues

The mechanical properties of the hydrogels were evaluated by micro-compression tests using a microsquisher. Stress-strain curves were generated to demonstrate densification trends and to compare compression moduli across major SG tissues and soy-based hydrogels (Fig. 3a,b). The linear region, 0–0.05 strain ($\mu\text{m}/\mu\text{m}$), was selected as it reflects the elastic behavior of the samples. Fig. 3a presented the stress-strain profiles of human SGs obtained from multiple donors ($n = 3$). The results showed that the compression moduli of submandibular glands (SMG) and sublingual glands (SLG) are closely aligned, approximately 0.7

± 0.5 kPa and 0.8 ± 0.4 kPa, respectively, while parotid glands exhibited a significantly lower modulus (0.2 ± 0.1 kPa), demonstrating that parotid glands were considerably softer than the other major glands ($p < 0.05$). Fig. 3b represented the stress-strain curves of soy-based hydrogels with multiple SPI concentrations. The control hydrogel (A2G7) exhibited the highest compression modulus (4.6 ± 1.7 kPa). Incorporation of SPI resulted in progressively lower moduli, ranging from 3.01 ± 1.7 kPa for S0.5A2G7, 1.1 ± 0.4 kPa for S1A2G7 and 1.01 ± 0.7 kPa for S2A2G7. The results indicated an inverse relationship between SPI concentrations and hydrogel stiffness (Fig. 3b). Fig. 3c revealed that the control hydrogel (A2G7) has a significantly higher compression modulus compared to soy-based hydrogels and human SG tissues ($p < 0.05$). Notably, although the S1A2G7 and S2A2G7 groups displayed a slightly higher compression modulus compared to native tissues, the differences were not significant ($p > 0.05$), suggesting mechanical compatibility between these soy-derived hydrogels and human SG tissues.

HuSMG Cells and Primary Salivary Functional Units (SFU) Can Reorganize to Form 3D Spheroids/Organoids in Soy-Based Hydrogels

Following the physical and chemical characterization of the hydrogels, we continued to evaluate their ability to

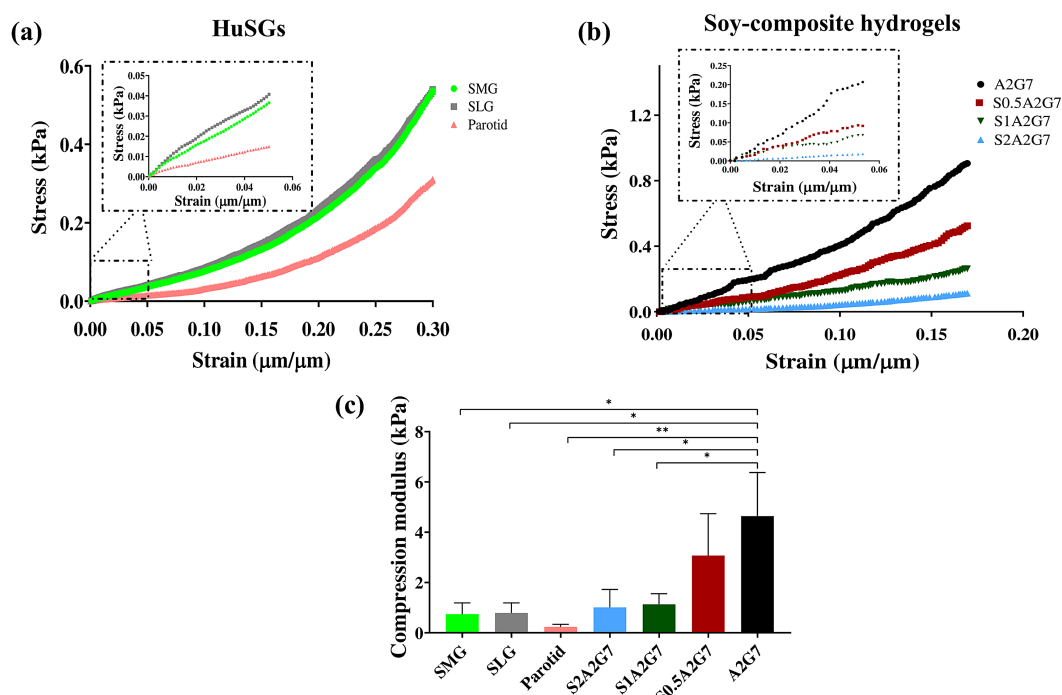


Fig. 3. Micro-compression tests of human SGs and soy-based hydrogels. (a) Stress-strain curves of three major human salivary glands: submandibular glands (SMG), sublingual glands (SLG) and parotid glands. The inserted box indicates the linear region of the stress-strain curve at low strains (0–0.05). (b) Stress-strain curves of hydrogels with different SPI concentrations. The inserted box highlights the linear region of the curves at low strains (0–0.05). (c) Comparison between compression moduli of human SGs and soy-based hydrogels. The compression moduli are calculated in the low-strain region (0–0.05). One-way ANOVA with Tukey's post hoc corrections was performed for data analysis. Data were presented as mean $\pm$ SD, $n \geq 5$, n.s., not significant; *: $p < 0.05$, **: $p < 0.01$. The figure was created by GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA).

support the attachment, proliferation, and structural organization of two different cell types: HuSMG cells and human-derived primary SG cells. Live/dead assays and confocal microscopy imaging were conducted to monitor cell viability and spheroid formation in the hydrogels over 4 weeks. In parallel, cellular metabolic activity over time was assessed using the WST-8 assay.

First of all, we tested our material with the HuSMG immortalized cell line, which was developed and characterized in our lab [31]. For benchmarking, the cells were also cultured in Matrigel, a widely used 3D matrix, to compare its performance with soy-based hydrogels. In Fig. 4a, early signs of small spheroid formation were observed within 7–14 days across all the groups. Cells in Matrigel® demonstrated significantly enhanced spheroid growth, forming much larger structures by the end of the first week. This trend continued to exhibit more rapid growth and cellular density by day 14. While cells in Matrigel® could form larger spheroids, their structures were significantly more irregular. On the other hand, spheroids formed in soy-based matrices were smaller but exhibited more organized, compact structures, potentially due to the differences in matrix composition and scaffold mechanical properties. Live/dead images (Fig. 4a) on Days 21 and 28 revealed

distinct differences in spheroid sizes among the groups ($p < 0.05$). Although spheroids in Matrigel® continued to increase in size over time, a substantial number of dead cells (stained red) accumulated in the spheroid center. This suggested a necrotic core in large spheroids, likely caused by limited nutrient and oxygen diffusion. In contrast, spheroids formed within soy-based hydrogels, particularly the S2A2G7 group, despite being smaller, maintained high viability with minimal central cell death. Metabolic activity, assessed by the WST-8 assay (Fig. 4b), was used as an indicator of cell proliferation. On Day 14, cells embedded in Matrigel® exhibited the highest metabolic activity, approximately 1.5 times that of the other groups, but this trend declined sharply thereafter, coinciding with an increase in core necrosis. By the end of the culture period, no significant differences in metabolic activity were observed among the groups ($p > 0.05$). The S2A2G7 group exhibited a consistently sustained increase in cellular metabolic activity, which is comparable to that of Matrigel® in long-term culture ($p > 0.05$). At the end of the culture period, despite the large spheroid sizes, the majority of cells inside Matrigel® were non-viable, resulting in approximately 50% viability. In contrast, the S1A2G7 and S2A2G7 groups maintain over 90% cellular viability after one month (Fig. 4c). Among

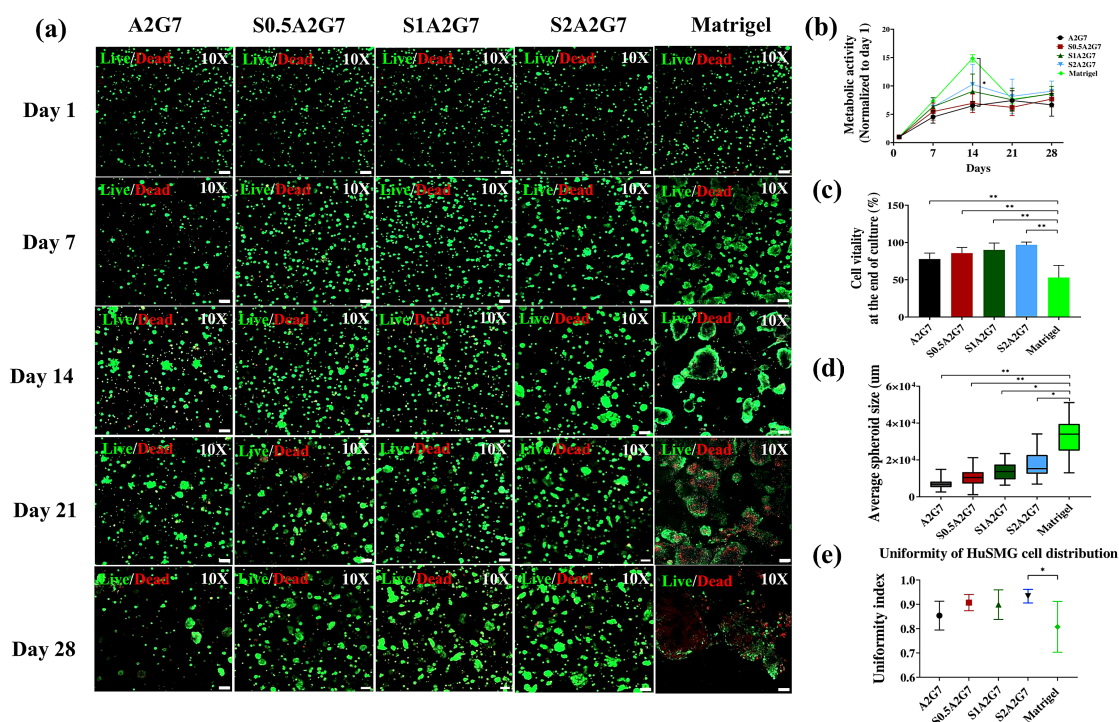


Fig. 4. HuSMG cell proliferation and spheroid formation in soy-based hydrogels. (a) Live/dead assay using confocal microscope imaging to evaluate cell morphology and spheroid formation in 5 groups: A2G7 (control group), S0.5A2G7, S1A2G7, S2A2G7 and Matrigel for 28 days. Green: live cells, red: dead cells. Magnification/scalebar: 10×/100 μm. (b) Metabolic activity of the spheroids by the WST-8 assay. (c,d) Cell viability (live cells/total number of cells) and spheroid sizes at the end of the culture period (on Day 28). (e) The uniformity of HuSMG distribution within the soy-composite hydrogel. One-way ANOVA followed by Bonferroni's post hoc test was conducted for data analysis. Data were presented as mean ± SD, n ≥ 5, *: $p < 0.05$, **: $p < 0.01$. The figure was created by GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA).

the soy-based hydrogel formulations, S2A2G7 supported the formation of the largest spheroid, with an average area of $16,358.9 \pm 7241.5 \mu\text{m}^2$ compared to the control group A2G7, exhibiting an average spheroid area of $7020.7 \pm 2672.5 \mu\text{m}^2$ (Fig. 4d). The uniformity of HuSMG cell distribution was quantified and presented in Fig. 4e. All the hydrogel groups exhibited relatively high uniformity indices (>0.8), suggesting homogeneous cell distribution within the 3D constructs. Although there was no significant difference in cell distribution across the hydrogel formulations, we observed that cells embedded in S2A2G7 hydrogel were distributed more uniformly than those in Matrigel, with a statistically significant increase in the uniformity index ($p < 0.05$).

HuSMG Spheroids in Soy-Based Hydrogels Exhibited Strong Expression of Epithelial Cell-Specific Markers, Including Ductal, Acinar and Myoepithelial Cells

Following the establishment of HuSMG spheroid models in hydrogels containing different SPI concentrations, we identified S2A2G7 as the formulation providing the most favorable microenvironment for long-term cell proliferation and reorganization. Based on these observations, we decided to continue using the S2A2G7 hydrogel

formulation for subsequent experiments. Additionally, as observed in the Live/Dead assay, cells in the soy hydrogel formed spheroid structures at a later stage of culture (day 21 to 28), while spheroids in Matrigel® developed earlier and exhibited substantial cell death in the cores by day 21 (Fig. 4a). This would restrict correct comparisons between the two hydrogel systems. Therefore, Matrigel® was used solely as a reference control for benchmarking cellular viability and proliferation in our study and was excluded from subsequent phenotypic expression and functional assays. Instead, the non-soy group (A2G7) was used as our control to ensure a more comparable evaluation in our further experiments.

To further validate the physiological behaviors of the HuSMG spheroids cultured in S2A2G7 hydrogel, we examined the expression of key cellular biomarkers previously analyzed in two-dimensional (2D) monolayer and 3D culture systems [36]. Immunofluorescence analysis revealed that the spheroids maintained elevated levels of key lineage-specific markers, including the epithelial tight junction protein (ZO-1), acinar cell-specific water channel (AQP5), ductal cell-specific marker (CK7) and myoepithelial cell marker (αSMA) (Fig. 5). The levels of phenotype expression in S2A2G7 were significantly higher than those

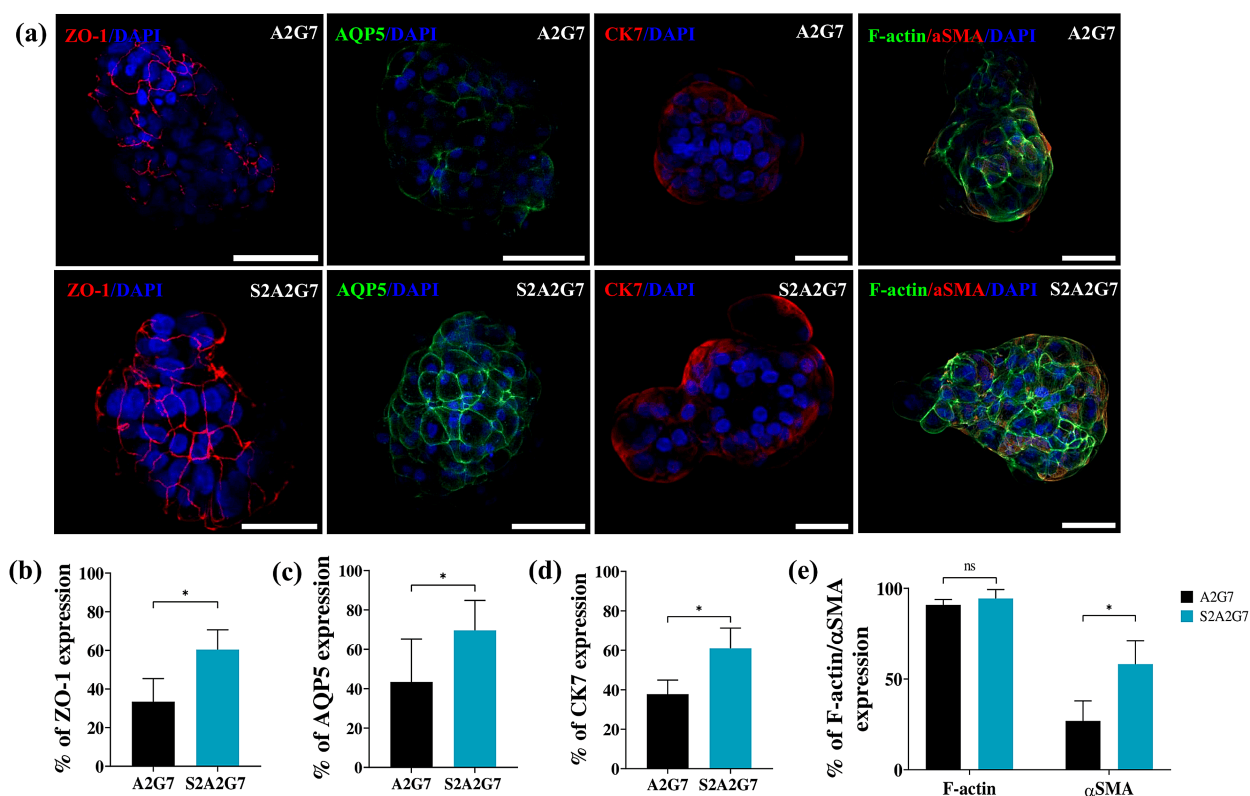


Fig. 5. HuSMG spheroids in the soy-based group exhibit enhanced expression of SG tissue-specific biomarkers. (a) Immunofluorescence staining of HuSMG spheroids at day 21–28 showing stronger expression of epithelial tight junction protein (ZO-1), acinar cell markers (AQP5), ductal cell markers (CK7), cytoskeletal structure (F-actin) and myoepithelial marker (α SMA), respectively, compared to the non-soy group. Magnification/Scale: $20\times/100\ \mu\text{m}$. (b–e) Quantification of the positive expression of these markers in HuSMG spheroids. Statistical analysis was performed using a two-tailed Student's *t*-test to compare the percentage of positive expression. Data were presented as mean $\pm$ SD ($n \geq 5$). n.s., no significant; *: $p < 0.05$. The figure was created by GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA).

in the non-soy hydrogel group ($p < 0.05$), confirming the structural diversity and epithelial retention of the spheroids in the soy-based hydrogel over an extended period. The distribution of F-actin was also evaluated to assess structural organization and epithelial polarity of the spheroids. The staining concentrated along the cellular membrane lining the apical surface of the spheroids demonstrated a well-organized cytoskeletal structure and retained epithelial polarity. However, no significant differences in the overall structural integrity were observed between the S2A2G7 and A2G7 groups ($p > 0.05$).

Soy-Based Hydrogels Could Support Functional HuSMG Spheroids in Long-Term Culture

In native SG tissues, fluid secretion is one of the key physiological functions, critical to maintaining homeostasis in the oral cavity. Building on our immunofluorescence results confirming the presence of acinar-like HuSMG spheroids with an enhanced AQP5 marker, we next investigated their secretory functionality in soy-based hydrogel. Secretory potential was evaluated using a Fluo-4

calcium imaging assay to monitor the intracellular Ca^{2+} release patterns in response to pharmacological stimulation, including Carbachol (muscarinic receptor agonist), ATP (purinergic receptor agonist), Isoprenaline (β -adrenergic receptor agonist) and DPBS without Ca^{2+} and Mg^{2+} (negative control) (Fig. 6 and **Supplementary Fig. 2**). When DPBS was added, no detectable intracellular calcium movement was observed, as the fluorescence signal remained flat over time (Fig. 6a,e, and **Supplementary Fig. 2a**). On the other hand, ATP stimulation induced a moderate and sustained increase in Ca^{2+} influx. ATP acted on purinergic receptors, triggering intracellular calcium signalling [37]. The moderate response to ATP suggested that spheroids possess functional purinergic receptors and could facilitate physiological fluid secretion (Fig. 6b,f, and **Supplementary Fig. 2b**). Additionally, Carbachol stimulation elicited a sharp and transient spike that briefly returned to baseline. This change indicated intense activation of muscarinic receptors, which mimicked the physiological role of acetylcholine in native SG (Fig. 6c,g, and **Supplementary Fig. 2c**). Finally, the stimulation of Isoprenaline re-

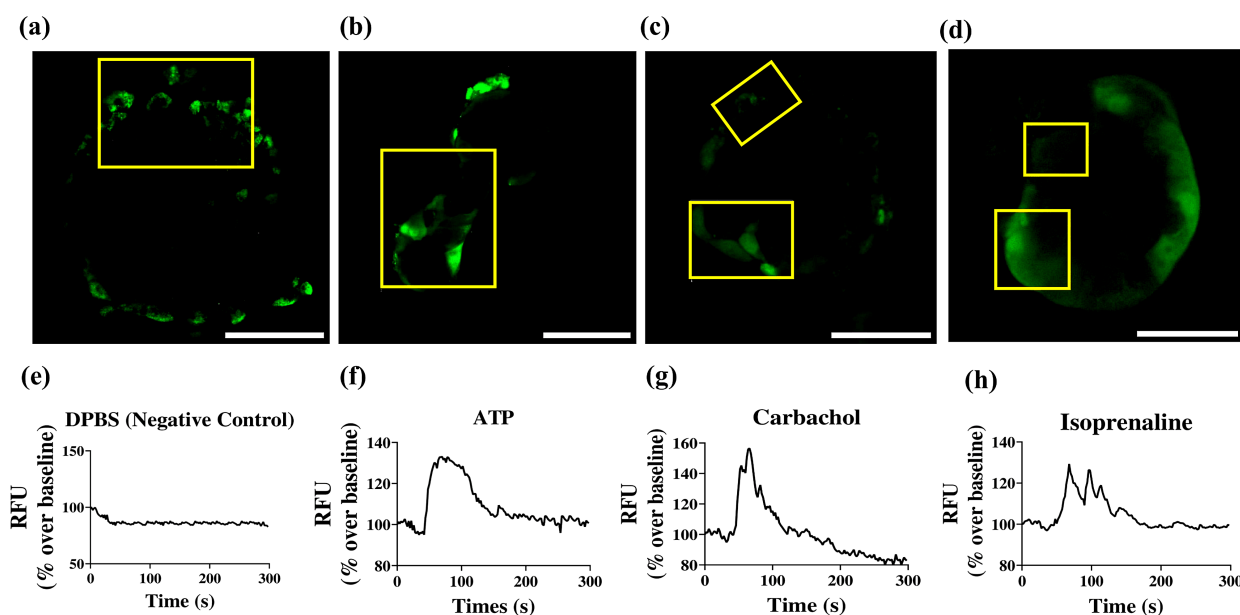


Fig. 6. Functional assessment of HuSMG spheroids cultured in soy-based hydrogel. (a–d) Spheroids were stimulated with DPBS (Ca^{2+} and Mg^{2+} free) as a negative control, ATP (100 μM), Carbachol (100 μM) or Isoprenaline (100 μM), respectively. Images are part of videos in **Supplementary Fig. 2**. Magnification/Scale: $20\times/100\ \mu\text{m}$. (e–h) Representative plots illustrating Fluo-4 peaks obtained by calcium influx in HuSMG spheroids tested with different agonists. Data are presented as mean $\pm$ SD ($n \geq 5$). The figure was created by GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA).

sulted in moderate elevations of intracellular calcium levels, followed by a gradual decline (Fig. 6d,h and **Supplementary Fig. 2d**). This pattern reflected the kinetics of the β -adrenergic receptor-mediated signalling pathway. In general, these excitation patterns demonstrated that long-term-cultured HuSMG spheroids in soy-based hydrogel can retain functional β -adrenergic, muscarinic, and purinergic receptors. These results highlighted the ability of soy-based hydrogel to support the functionality of mature HuSMG spheroids and helped them retain key SG biomarkers in 3D culture.

Soy-Based Hydrogels Serve as a 3D Culture Platform That Promotes Cell Self-Organization and Maintains Functional Markers in Human Primary Salivary Functional Units in Long-Term Culture

Due to the limited capacity of immortalized HuSMG cell lines to replicate the physiological characteristics of human tissue, we used human primary SG cells to evaluate the bio-performance of soy-based hydrogels in a more physiologically relevant context. For this purpose, we utilized human salivary functional units (SFUs), which were derived from the native salivary glands of multiple donors and were fully developed and characterized in our previous study [38] as a physiological model to assess the biocompatibility of our soy-based material.

Live/dead confocal imaging (Fig. 7a) demonstrated that SFUs cultured in a soy-based hydrogel could form com-

pact and well-organized structures, which developed into larger organoids over 28 days. Compared to the non-soy group, SFUs in the S2A2G7 hydrogel exhibited a higher proliferation rate over a long-term culture period ($p < 0.05$). Consistent with Live/Dead results, metabolic activity, measured by the WST-8 assay (Fig. 7b), revealed a significant rise in cell proliferation in the soy group over 21 days ($p < 0.05$). At the end of the culture period, organoids in the soy-based group displayed greater cell viability and larger organoid structures (shown in Fig. 7c,d), with an average area of $21,119.7 \pm 4722.9\ \mu\text{m}^2$ in S2A2G7 gel, compared to $8500.2 \pm 3969.9\ \mu\text{m}^2$ in the control group ($p < 0.05$). The uniformity of cell distribution was assessed using the uniformity index, which is approximately 0.7 ± 0.1 for the control group and around 0.8 ± 0.07 for the soy-composite counterpart (Fig. 7e). Although the soy hydrogel shows a slightly higher uniformity index, no significant difference in cell distribution was observed between the two groups ($p > 0.05$).

To assess the functional responses in the organoids, a Fluo-4 calcium imaging was performed, revealing distinct response features across different agonists (Fig. 7f). Similar to the results with HuSMG spheroids, the SFU organoids exhibited no detectable calcium influx in DPBS (Fig. 7g). In contrast, they exhibited a robust response to the ATP agent with a sharp and transient increase in intracellular Ca^{2+} influx in the cells (Fig. 7h). Carbachol, similarly, to our previous experiment, induced a more remarkable

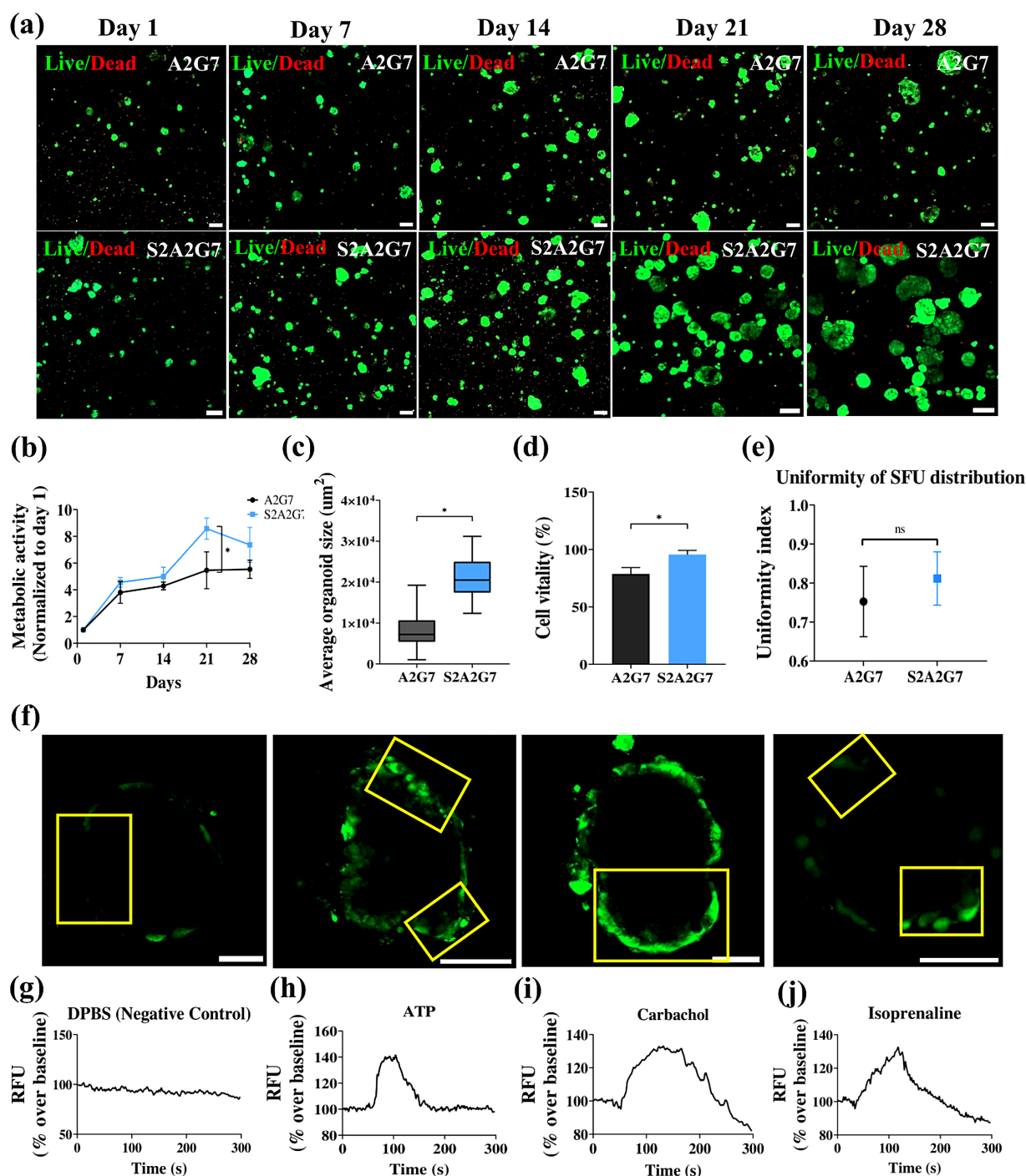


Fig. 7. Three-dimensional culture of human primary SG cells in soy-based hydrogels. (a) Representative Live/dead images of SG organoids cultured in soy-based hydrogels for 28 days. Magnification/Scale: $10\times/100\ \mu\text{m}$. (b) Metabolic activity of SG organoids assessed by WST-8 assays. (c,d) Average organoid size and organoid viability in soy-based hydrogel at the end of the culture period, respectively. (e) The uniformity of SFU distribution within the soy-composite hydrogel. (f) Organoids are stimulated with DPBS (Ca^{2+} and Mg^{2+} free) as a negative control, ATP ($100\ \mu\text{M}$), Carbachol ($100\ \mu\text{M}$), or Isoprenaline ($100\ \mu\text{M}$), respectively. Images are part of the videos in **Supplementary Fig. 3**. (g–j) Representative plots illustrating Fluo-4 peaks obtained by calcium influx in SG organoids with different agonists. Statistical analysis was performed using a two-tailed Student's *t*-test to compare the percentage of positive expression. Data were presented as mean $\pm$ SD ($n \geq 5$). Magnification/Scale: $20\times/100\ \mu\text{m}$. The figure was created by GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA).

increase in calcium influx with a longer duration through muscarinic receptor stimulation (Fig. 7i). Finally, Isoprenaline elicited a moderate and gradual calcium response of β -adrenergic receptor activation, consistent with the calcium assay results of HuSMG spheroids (Fig. 7j). Taken together, these data confirmed that SG organoids could retain functional integrity and exhibited physiologically relevant signalling even after 28 days of 3D culture in a soy-based environment.

The organoids from human SFUs cultured in soy-based hydrogel for 28 days exhibited significantly more robust expression of epithelial markers, such as AQP5 (secretory function), AMY1 (amylase secretion), α SMA (myoepithelial cells), CK7 (luminal ductal cells), ZO-1 (epithelial tight junction marker), as well as progenitor markers, including CK5, CK14 and CD117 (basal/ductal progenitor cells), compared to those in the control group (Fig. 8) ($p < 0.05$). The expression of AQP5 was localized along the apical membrane of the cells, suggesting the presence of secretory acinar-like cells, together with positive AMY1 (α -amylase) expression, confirming the mature secretory functions of the organoids. Additionally, α SMA, a contractile component that supports glandular architecture, was also predominantly found at the periphery of the organoids. The increased expression of the ductal marker (CK7) and basal progenitor markers (CK5/CK14) in organoids indicated the presence of a heterogeneous population of luminal and basal cells, while the enhanced CD117 expression suggested the population of stem-like cells in human SG organoids. The expression levels of F-actin and E-Cad were similar between the two groups, indicating that overall epithelial integrity and cytoskeletal organization were relatively comparable ($p > 0.05$).

Discussion

Scaffold fabrication, along with cell engineering and growth factors, plays a critical role in the success of tissue engineering. Despite substantial advancements in fabricating *in vitro* 3D environments to emulate native tissue architecture, critical challenges persist in optimizing physicochemical properties, as well as ensuring biocompatibility and tissue support of the new biomaterial. In this study, we have developed a composite soy-based hydrogel as a 3D biomimetic platform that can support cellular proliferation, migration, and structural reorganization for SG tissue engineering. We have successfully evaluated our hydrogel using both a human SG cell line (HuSMG) and primary cells from SFUs in long-term culture, demonstrating its potential as a robust scaffold for glandular regeneration.

The FTIR results (Fig. 1) confirm the presence of characteristic functional groups of gelatin, alginate and SPI, validating the complete integration of these components within the matrix. Similar characteristic peaks for the individual components in the hydrogel have been reported in previous studies [19,22,34,39]. These earlier reports

attribute the structural integrity of gelatin-, alginate-, and SPI-based systems primarily to non-covalent interactions, namely hydrogen bonding and electrostatic forces [40]. The increasing soy concentration in the formulation enhanced amide-related peaks, particularly within the regions around 2900 cm^{-1} and $1500\text{--}1600\text{ cm}^{-1}$, suggesting physical entrapment and effective incorporation of SPI within the matrix without significant alteration of other functional groups. Similar observations have been documented in polyvinyl alcohol/SPI composite hydrogels, where the retention of protein secondary structures, along with polysaccharide interactions, was confirmed by FTIR analysis [21].

Following up, we continued to characterize the structural, mechanical and degradation properties of our soy-based hydrogel. SEM analysis in Fig. 2b revealed that the mean pore areas ranged from 2500 to $6500\text{ }\mu\text{m}^2$, corresponding to approximately $56\text{--}90\text{ }\mu\text{m}$ in diameter across all groups. Pore size is a critical regulator of cell behaviour, as pore-mediated mechanical constraints can regulate the cytoskeletal organization and force transmission to the nuclei, thereby altering gene expression and cellular morphology [41]. Akay *et al.* [42] reported that cells concentrated more densely in the scaffolds with relatively smaller pores, while rapid cellular infiltration occurred more frequently inside the large porous scaffolds. Average pore sizes of $40\text{--}100\text{ }\mu\text{m}$ have been shown to facilitate more efficient nutrient diffusion, promote better cell adhesion, proliferation and migration [43]. Additionally, other authors observed that smaller pore sizes could impede the diffusion of focal adhesion proteins into the cell membrane, restrict cellular movement and suppress key regenerative behaviors [44]. Optimal pore sizes of biomaterials are highly tissue-specific, ranging from $5\text{--}15\text{ }\mu\text{m}$ for fibroblasts [45], $20\text{ }\mu\text{m}$ for hepatocytes [45], $60\text{--}150\text{ }\mu\text{m}$ for smooth muscle cells [46], and $500\text{--}750\text{ }\mu\text{m}$ for optimal osseointegration in bone tissue engineering [47]. In the context of salivary gland epithelial cells, no studies have specifically defined the optimal pore diameter for cell growth. However, it has been reported that pore sizes between $20\text{--}125\text{ }\mu\text{m}$ are favourable for epithelial cell migration and organization [48], which matches the pore size findings in our study. We also observed that increasing the SPI concentration resulted in larger pore sizes in the hydrogel structure, which is advantageous for nutrient diffusion, cell migration, and spheroid formation. In contrast, the non-soy formulation (A2G7) exhibited significantly smaller pores, which was associated with limited cell proliferation and spheroid development. Although the pore size of the S2A2G7 hydrogel decreased significantly after 14 days of incubation ($p < 0.05$), likely due to partial matrix degradation and structural remodelling, the hydrogel still maintained larger pores than the other formulations. These pores remained sufficiently large to support cell proliferation, migration, and long-term spheroid growth.

Regarding the swelling and degradation behaviour in Fig. 2c, the gradual and controlled swelling behavior

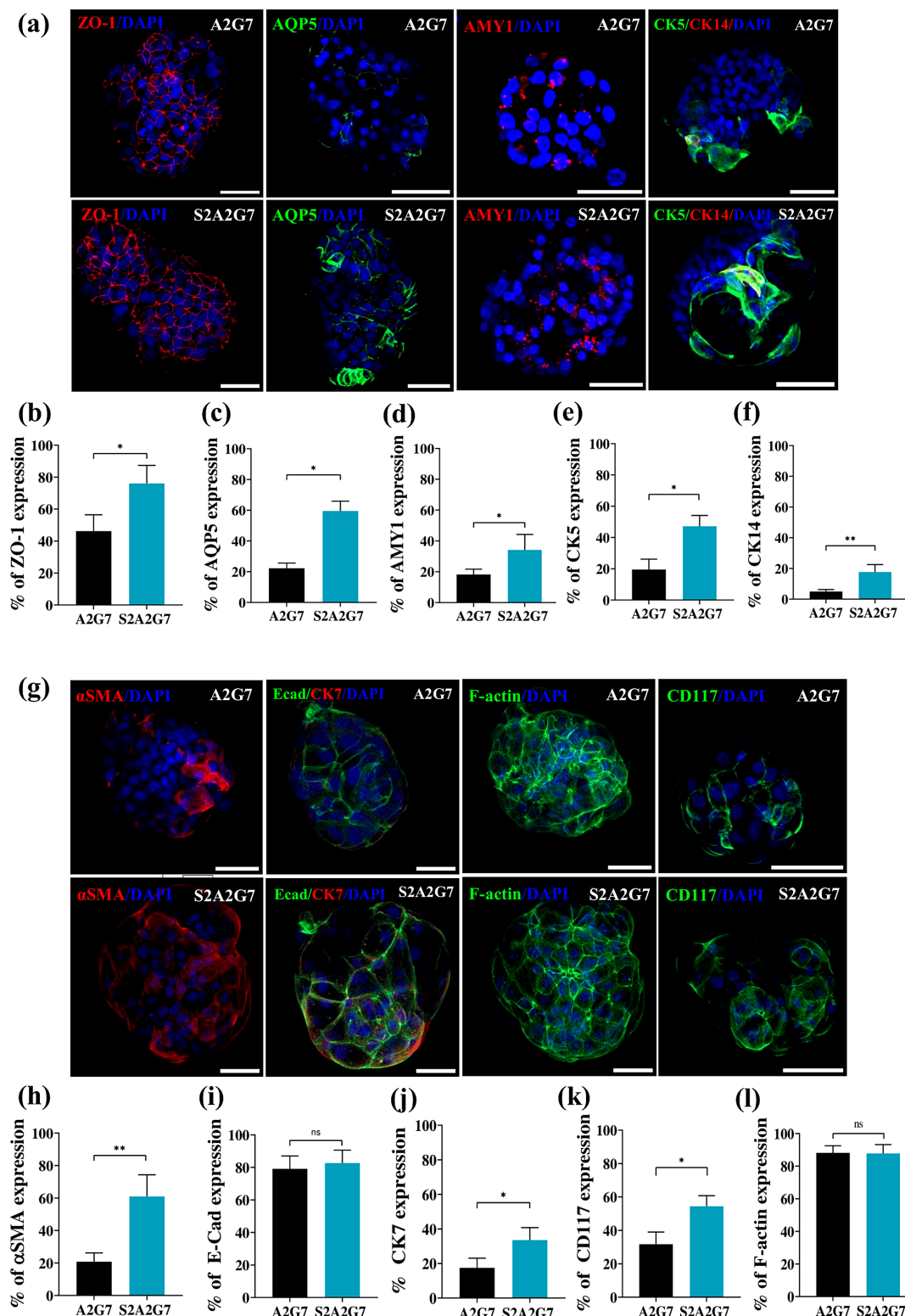


Fig. 8. Immunofluorescence staining of human primary SG cells with different markers in soy-based hydrogels. (a,g) Confocal images of SG markers (ZO-1, AQP5, AMY1, CK5/CK14, αSMA, E-Cad/CK7, F-actin, and CD117) expressed on salivary gland organoids. (b–f, h–l) Quantification of positive expression of these biomarkers on human organoids. Magnification/Scale: 20×/100 μm. Statistical analysis was performed using a two-tailed Student's *t*-test to compare the percentage of positive expression. Data were presented as mean ± SD (*n* ≥ 5). n.s., no significant; *, *p* < 0.05, **, *p* < 0.01. The figure was created by GraphPad Prism version 10.0 (GraphPad Software, San Diego, CA, USA).

of the soy-based hydrogel is beneficial for its application in SG engineering, as it will inhibit sudden mechanical stress on soft glandular tissue and allow sustained hydration [49]. Toward the end of the incubation period, degradation became more evident in soy-based groups, especially in S2A2G7, which experienced the highest weight loss, approximately 20% by day 21. A similar trend has also been observed by Tansaz *et al.* [19], who reported that alginate/SPI films with higher SPI concentrations underwent greater degradation compared to the pure alginate counterpart. Such partial degradation of the naturally derived hydrogel over several weeks is desirable, as it allows for gradual matrix resorption and provides a proper replacement by new tissue without compromising structural integrity in the early stages of soft tissue regeneration [50].

From the mechanical test, soy-based hydrogels also exhibited tunable stiffness, with an approximate compression modulus of 1–3 kPa; there was a reverse correlation between the compression modulus and the concentration of SPI, making S2A2G7 significantly softer than the control group A2G7 (Fig. 3c). The reduction in stiffness of soy-based hydrogels is likely due to the disruption of intermolecular crosslinking and the increased porosity of the matrix. Notably, the S2A2G7 formulation exhibited compression moduli comparable to those of the native SG ($p > 0.05$). Such compatibility in scaffold stiffness may help minimize the mechanical mismatch, thereby maintaining the physiological function of the cells and promoting tissue integration [51–53]. Miyajima *et al.* [54] observed that softer substrates could promote better cell differentiation, phenotypic retention and branching morphogenesis for *ex vivo* SG embryogenic tissue. In contrast, stiffer hydrogels could cause the cells to spread out, leading to an increase in focal adhesion of progenitor cells, higher integrin clustering, disruption of basal polarity, and inhibition of lumen formation and acinar cell reorganization [55]. Therefore, the mechanical properties of the S2A2G7 formulation, which closely resemble those of the native SG tissue, provide a favourable microenvironment to support SG tissue engineering.

Our present study focused on static mechanical characterization to compare the hydrogel's compressive properties with those of salivary gland tissue. We aimed to develop a 3D microenvironment from soy-based hydrogel that supports SG cell viability, differentiation and restores secretory functions. Since salivary glands are not load-bearing tissues, like cartilage or tendon, and they are exposed only to relatively mild indirect mechanical forces, static mechanical testing was considered sufficient for this proof-of-concept study. Nonetheless, dynamic loading tests, including dynamic mechanical analysis (DMA) or cyclic compression, can provide valuable insight into the hydrogel's viscoelastic behaviour and mechanical stability and better capture the cyclic forces experienced by salivary gland tissue physiologically. Although beyond the scope

of the present work, such experiments will be considered in future investigations, especially for *in vivo* applications.

After confirming the physicochemical properties of the soy-based material, we evaluated its biocompatibility and biological behaviours using the human SG cell line (HuSMG), previously developed by our lab [31], and human primary cells. First, we utilized HuSMG cells to compare the performance of our hydrogels with that of Matrigel®, the gold standard in the current market. Our results, as shown in Fig. 4, indicated that while Matrigel® could promote rapid spheroid formation and growth, the resulting spheroids exhibited irregular architecture and necrotic cores in long-term culture, possibly owing to limited nutrient and oxygen diffusion. Additionally, Matrigel®'s low stiffness (443 Pa) [56] makes it difficult to maintain the material's structure, causing it to be highly susceptible to deformation under cell traction forces after weeks of culture. In contrast, soy-based hydrogels could facilitate more compact and organized spheroids, without necrotic cores at the end of the culture period. The metabolic assay confirmed sustained cell viability in the soy-derived groups for up to one month, with cell viability in the S2A2G7 group at the end of the culture period being comparable to that in the Matrigel® group. Although the precise mechanisms underlying the beneficial effects of SPI on SG cell culture have not been widely investigated, previous studies have demonstrated its capacity to provide excellent support for the attachment and proliferation of various cell types, including fibroblasts, human endothelial cells, and osteoblasts [19,22]. One possible explanation is that the gradual biodegradation of SPI exposes cell-adhesive peptide motifs, such as Arginine-Glycine-Aspartic (RGD)-like sequences, which help to enhance cellular attachment, even under serum-free culture conditions [57]. Glycine, a proteolytic degradation amino acid of glycinin—one of the major proteins in SPI—is an important component for collagen triple-helix formation and can provide substrates for ECM synthesis and remodelling. SPI is abundant in hydrophilic amino acids that bind to water molecules, contributing to the formation of highly hydrated hydrogels favourable for nutrient and oxygen diffusion throughout the construct [58]. Additionally, the mechanical properties of the soy-based hydrogel are also a contributing factor in regulating cellular behaviour. Cells sense and respond to substrate stiffness through integrin-mediated mechanotransduction pathways, which significantly affect the cytoskeletal organization, cellular polarity, and proliferative capacity [58]. From our findings, the incorporation of SPI into the hydrogel platform can alter the mechanical characteristics of the matrix, making it more representative of the soft tissue microenvironment and therefore potentially enhancing the retention of epithelial phenotype and functionality of salivary gland tissue in long-term culture. Following that, we applied the 2% soy-based hydrogel formulation (S2A2G7) to human primary cells using SFUs and observed consistent outcomes. The S2A2G7 hydro-

gel provided a supportive microenvironment for human SG organoids over 28 days, enabling enhanced organoid formation and sustained metabolic activity. This prolonged cell viability and reorganization highlights the potential of our novel hydrogel as a physiologically relevant matrix for SG engineering. Uniformity indices of both HuSMG and SFU distributions were assessed to determine the ability of the hydrogels to maintain homogeneous cellular dispersion within the matrix, with values closer to 1 indicating more homogeneous cell distribution. In both cell types, these values across the hydrogel formulations were relatively high (>0.8), especially in the S2A2G7 group (Figs. 4e and 7e). The enhanced uniformity observed in S2A2G7 compared to Matrigel in Fig. 4e can be attributed to differences in the physicochemical properties of the matrices. Matrigel exhibited weaker mechanical properties and temperature-dependent gelation behaviour, causing the cells to be unevenly distributed before complete gelation [56]. In contrast, the soy-based composite hydrogel network can provide more controlled gelation kinetics and matrix architecture, thereby facilitating a more uniform spatial dispersion of embedded cells. Irregular cell suspension may result in localized areas of higher cellular density, leading to uneven nutrient gradients and non-uniform cell proliferation [59]. Consistent with this concept, previous studies have demonstrated that hydrogels capable of evenly encapsulating cells can enhance cellular viability, proliferation and long-term organoid formation [60].

Our immunofluorescence staining in Figs. 5 and 7 showed that cells in the S2A2G7 hydrogel maintained the epithelial phenotype and functional maturation after weeks of culture. In HuSMG spheroids, epithelial-specific markers, including ZO-1, AQP5, CK7, and α SMA, were more upregulated than those in the control group ($p < 0.05$). The tight junction protein ZO-1 presented its characteristic distribution at the intercellular junctions of the adjacent cells. The strong expression of ZO-1 suggested robust intercellular connections and the establishment of apical-basal polarity [61,62]. AQP5 is a membrane-bound water channel protein predominantly found in the apical membrane of acinar cells, helping to facilitate transcellular water transport. The increased expression of AQP5 in our HuSMG spheroids indicated the presence of predominantly acinar-like cells, confirming acinar maturation associated with the fluid secretion function. Additionally, the upregulation of CK7 and α SMA indicates the presence of certain ductal and myoepithelial cell populations. CK7 is a ductal epithelial marker, predominantly expressed in luminal ductal cells, whose expression indicates the retention of the ductal lineage, while the α SMA marker denotes the functional myoepithelial component, serving a critical role in contractile function for fluid expulsion during cellular stimulation [63]. These patterns highlight the potential of the spheroids to recapitulate key functions of SGs [64,65]. Similar results were also observed in SFU organoids (Fig. 8) with

upregulation of epithelial markers (AQP5, CK7, α SMA, AMY1) and progenitor markers (CK5, CK14, CD117) in the S2A2G7 hydrogel. CK5 is a cytokeratin marker in ductal basal progenitor cells, which are essential for early regenerative responses [66]. In contrast, CK14, often expressed in active basal cells of stratified epithelia and myoepithelial cells, signifies a more differentiated basal cell population [67]. In our results, a dominant expression of CK5, but limited expression of CK14, was observed, suggesting the presence of a progenitor basal-leaning population rather than a fully developed basal phenotype. As CK5 and CD117 are often associated with progenitor cell populations, whereas the CK7 marker represents differentiated ductal cells, the enhanced expression of CK5, CK7, and CD117 indicated the coexistence of both progenitor and differentiated cell phenotypes within the hydrogel. This finding suggests a heterogeneous composition with regenerative potential in SG organoids. These marker expressions were evaluated at the end of the culture period (Day 28), indicating that the phenotypes were maintained up to 28 days of culture. Such preservation contributes to the organoid's capacity to recapitulate tissue architecture and key physiological features of native SG tissues. Nonetheless, further longitudinal studies are needed to evaluate the retention of these markers over longer culture time.

To further validate the functionality, we performed calcium assays on the SG cells (Figs. 6,7). The calcium signaling patterns observed in both cell types closely matched the native physiology of the SG, as reported in previous papers [68,69]. Fedirko *et al.* [37] reported that extracellular ATP triggers a biphasic calcium influx via two purinergic receptors: an initial transient rise through P2Y receptors and a sustained phase through P2X receptors. The calcium response is dose-independent and requires both channels for the full effect. In our study, both HuSMG spheroids and primary SG organoids exhibited a moderate, sustained Ca^{2+} increase upon ATP stimulation, aligning strongly with the authors' observations [37]. In addition, Carbachol stimulation induced a sharp and intense intracellular Ca^{2+} elevation in both cell types, reflecting robust muscarinic activation through IP3-mediated Ca^{2+} release from the endoplasmic reticulum (ER). The result was consistent with prior literature, which reported that muscarinic receptor stimulation elicited rapid and intense ER calcium influx [68,70]. Following this initial spike, store-operated calcium entry (SOCE) kicks in to sustain calcium secretion [70], and this pattern can be observed more clearly in the Carbachol-induced calcium response of primary SG cells. In contrast to Carbachol and ATP stimulation through muscarinic and purinergic pathways, Isoprenaline induced a more gradual and moderate calcium peak, owing to the β -adrenergic receptor-mediated signaling. The delayed and prolonged calcium elevation in our experiment confirms the presence of functional β -adrenergic receptors in SG organoids after long-term culture in the soy-based hydro-

gels. From our results, SG organoids cultured in soy-based hydrogels showed a clear response to purinergic, muscarinic and β -adrenergic stimulations with distinct calcium influx patterns. The trends demonstrated the activation of key receptor-mediated pathways that closely mimicked those in native tissue. This functional responsiveness, combined with sustained viability and structural organization, highlights the potential of soy-based hydrogels as a biomimetic platform for long-term salivary gland tissue engineering and regenerative applications.

Conclusions

While 3D cell culture systems have been previously developed, this study introduces one of the first scalable, plant-protein-derived hydrogels tailored for salivary gland cell regeneration, with the potential to become a more sustainable alternative to Matrigel®. Our preliminary results demonstrate that incorporating SPI into the hydrogel matrix can modulate the structural architecture and mechanical properties, resulting in a softer hydrogel network. This modification enhances biological relevance by better mimicking the native ECM, improving biocompatibility, organoid formation and functional performance of SG cells. The long-term viability, cellular heterogeneity, and specific lineage marker expression of SG organoids validate soy-based hydrogels as a promising biomimetic platform for 3D culture. Further studies are essential to systematically optimize their physicochemical properties and to evaluate cellular responses under pathological conditions, such as radiation induced damage. This platform could potentially be applied to *in vitro* and *ex vivo* drug screening, as well as disease modelling studies of complex salivary gland tissue in future studies.

List of Abbreviations

2D, two-dimensional; 3D, three-dimensional; A, alginate; AMY1, α -amylase; AQP5, aquaporin 5; ATR, attenuated total reflectance; CaCl_2 , calcium chloride; CK14, cytokeratin 14; CK5, cytokeratin 5; CK7, cytokeratin 7; DMA, dynamic mechanical analysis; E-Cad, epithelial cadherin; ECM, extracellular matrix; ER, endoplasmic reticulum; F-actin, filamentous actin; FBS, fetal bovine serum; FDA, food and drug administration; FTIR, fourier-transform infrared spectroscopy; G, gelatin; HuSMG, human submandibular gland cells; IPN, interpenetrating network; PBS, phosphate-buffered saline; RGD, Arg-Gly-Asp; SD, standard deviation; SEM, scanning electron microscopy; SFU, salivary functional unit; SG, salivary gland; SOCE, store-operated calcium entry; SPI, soy protein isolate; UV, ultraviolet; ZO-1, zonula occludens-1; α SMA, alpha-smooth muscle actin; SLG, sublingual glands.

Availability of Data and Materials

All datasets generated or analyzed during the current study are included in this published article and its Supplementary Information. Additional experimental details and data used or analyzed in this study are available upon reasonable request.

Author Contributions

UMNC, JI, and SDT designed and conceptualized this work. UMNC collected and analyzed the data and drafted the manuscript. YZ helped with mechanical testing, including the experiment's methodology, validation, and review. CA helped with performing the FTIR experiment, interpretation and validation of data. UMNC, JI, YZ and CA contributed to the interpretation of data. AZ and JG performed the surgical procedures for tissue collection. MC and SDT supervised the paper. All authors read and agreed to the published version of the manuscript. All authors approved the final manuscript and agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics Approval and Consent to Participate

Use of human samples was approved and conducted according to the guidelines of the McGill Institutional Review Board (IRB), with study number A05-M62-05B. All the participants provided signed informed consent.

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Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.22203/eCM.v059a16>.

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