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Encapsulation of primary salivary gland cells in enzymatically degradable poly(ethylene glycol) hydrogels promotes acinar cell characteristics

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ABSTRACT

Radiation therapy for head and neck cancers leads to permanent xerostomia due to the loss of secretory acinar cells in the salivary glands. Regenerative treatments utilizing primary submandibular gland (SMG) cells show modest improvements in salivary secretory function, but there is limited evidence of salivary gland regeneration. We have recently shown that poly(ethylene glycol) (PEG) hydrogels can support the survival and proliferation of SMG cells as multicellular spheres *in vitro*. To further develop this approach for cell-based salivary gland regeneration, we have investigated how different modes of PEG hydrogel degradation affect the proliferation, cell-specific gene expression, and epithelial morphology within encapsulated salivary gland spheres. Comparison of non-degradable, hydrolytically-degradable, matrix metalloproteinase (MMP)-degradable, and mixed mode-degradable hydrogels showed that hydrogel degradation by any mechanism is required for significant proliferation of encapsulated cells. The expression of acinar phenotypic markers *Aqp5* and *Nkcc1* was increased in hydrogels that are MMP-degradable compared with other hydrogel compositions. However, expression of secretory acinar proteins *Mist1* and *Pip* was not maintained to the same extent as phenotypic markers, suggesting changes in cell function upon encapsulation. Nevertheless, MMP- and mixed mode-degradability promoted organization of polarized cell types forming tight junctions and expression of the basement membrane proteins laminin and collagen IV within encapsulated SMG spheres. This work demonstrates that cellularly remodeled hydrogels can promote proliferation and gland-like organization by encapsulated salivary gland cells as well as maintenance of acinar cell characteristics required for regenerative approaches. Investigation is required to identify approaches to further enhance acinar secretory properties.

Statement of Significance

Regenerative strategies to replace damaged salivary glands require the function and organization of acinar cells. Hydrogel-based approaches have shown promise to control cell function and phenotype. However, little is known about how specific parameters, such as the mechanism of hydrogel degradation (e.g., hydrolytic or enzymatic), influence the viability, proliferation, organization, and phenotype of salivary gland cells. In this work, it is shown that hydrogel-encapsulated primary salivary gland cell proliferation is dependent upon hydrogel degradation. Hydrogels crosslinked with enzymatically degradable peptides promoted the expression of critical acinar cell markers, which are typically downregulated in primary cultures. Furthermore, salivary gland cells encapsulated in enzymatically- but not hydrolytically-degradable hydrogels displayed highly organized and polarized salivary gland cell markers, which mimics characteristics found in native gland tissue. In sum, results indicate that salivary gland

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cells respond to cellularly remodeled hydrogels, resulting in self-assembly and organization akin to acini substructures of the salivary gland.

1. Introduction

Saliva is produced by secretory acinar cells, which are the predominant cell type within salivary glands. The acinar cells are arranged in clusters surrounded by myoepithelial cells and drain saliva into a ductal tree leading to the oral cavity. Unidirectional movement of saliva requires proper apical and basolateral polarization of acinar and ductal cells within the gland.

For individuals with head and neck cancers, which are diagnosed in over 50,000 people in the U.S. annually, radiation therapy often causes extensive acinar cell loss. Subsequently, chronic dry mouth syndrome (i.e., "xerostomia") severely undermines oral health and function [1,2]. Though some radioprotective strategies are under development [3–8], current treatments for this major quality of life issue are only palliative [9]. Therefore, regenerative and/or radioprotective approaches for the salivary gland are critically needed.

Direct injection of primary mouse submandibular gland (SMG) cells into irradiated glands has been shown to partially restore gland function [10–12]. However, there is little evidence of acinar cell regeneration and the healing response is variable, likely due to poor cell localization and persistence in the gland. Regeneration strategies are further complicated by loss of the acinar cell phenotype *in vitro* [13–15].

Hydrogels have been used for localized cell delivery in numerous tissue engineering strategies [16–22]. Hydrogels provide highly controllable platforms to study the mechanistic effects of extracellular matrix (ECM) and soluble factors on encapsulated cell populations. Furthermore, hydrogels can be used to control cell localization and persistence simply through modulation of hydrogel degradation [18]. SMG cells have been cultured in several types of hydrogels derived from natural (e.g., Matrigel, fibrin, hyaluronic acid, and laminin) and synthetic (e.g., poly(ethylene glycol) (PEG)) materials [23–30]. Although natural materials support the viability, proliferation, and some SMG phenotypic characteristics such as apicobasal polarization, these hydrogels have limited chemical versatility and imbibe underlying biological cues [31], which could lead to undesirable side effects on cell phenotype and function. Matrigel suffers from significant batch-to-batch variability and potential tumorigenicity, limiting its use for cell transplantation [32,33]. In contrast, biologically inert and synthetically flexible PEG-based hydrogels provide control over the presentation of bioactive factors (e.g., adhesive ligands) and chemical and physical characteristics (e.g., degradability) of hydrogels [34–40].

We previously identified PEG hydrogels as a promising platform for primary salivary gland cell culture [28]. Specifically, we found that allowing SMG cell sphere formation prior to encapsulation and using thiol-ene versus chain-polymerized crosslinking promoted cell survival and proliferation for up to 14 days *in vitro*. Here, we have examined how different modes of PEG hydrogel degradation affect the proliferation, organization, and phenotype of encapsulated SMG cells.

2. Methods

2.1. PEG macromer synthesis

2.1.1. Materials

Dithiol-functionalized PEG (PEGDT, 3.4 kDa) was purchased from Laysan Bio, and 4-arm hydroxyl-functionalized PEG (PEG-OH, 20 kDa) was purchased from JenKem Technologies, USA. All other materials were purchased from Sigma-Aldrich unless specified otherwise. Synthesis of lithium phenyl-2,4,6-trimethylbenzoyl phosphinate (LAP) was performed as described previously [41].

2.1.2. 4-arm PEG-NH_2 synthesis

4-arm 20 kDa PEG-NH_2 was synthesized from a 4-arm 20 kDa PEG with hydroxyl end groups on each arm (PEG-OH) using end group substitution via a mesylate intermediate. 10 g of 4-arm 20 kDa PEG-OH was dissolved in 200 mL toluene and the solution was evaporated to ~100 mL by azeotropic distillation. The solution was cooled to room temperature, 50 mL of dichloromethane (DCM) was added, and the solution was placed on ice for 15 min with constant stirring. 4 M equivalents (meq) per OH of triethylamine (TEA) were added, followed by 4 meq per OH of methanesulfonyl chloride added dropwise. The solution was purged with N_2 gas, allowed to react on ice overnight, vacuum filtered to remove salt precipitates, and concentrated to 20–30 mL using a Rotorvapor® RII rotovap (Buchi) at 60 °C. Concentrated 4-arm PEG-mesylate was precipitated in 10x volume of ice-cold diethyl ether, collected via filtration, and dried overnight under vacuum. Amine functionalization of mesylate-functionalized 4-arm PEG was performed by dissolving the entire PEG-mesylate product in 300 mL of 30% NH_4OH, stirring for 3 days, and evaporating via exposure to atmosphere until the volume was reduced to ~100 mL (over ~4 days). The solution was raised to pH 13 using 1 M NaOH and 100 mL of DCM was added. After overnight separation, the aqueous fraction was extracted twice more and collected DCM was dried over anhydrous sodium sulfate. After filtration and concentration to 20–30 mL, the PEG product was precipitated in ~300 mL of ice-cold diethyl ether and collected via filtration. Confirmation of both mesylate and amine functionalization was determined by ^{1}H-NMR (>90% functionalization) performed on a Bruker Avance™ 400 MHz spectrometer: 4-arm PEG-mesylate (^{1}H NMR ($CDCl_3$): δ = 4.2 ppm (ether protons adjacent to mesylate group, 8H, singlet), 3.5–3.9 ppm (PEG ether protons, 1817H, multiplet)); 4-arm PEG-NH_2 (^{1}H NMR ($CDCl_3$): δ = 3.0 ppm (ether protons adjacent to amine group, 8H, singlet), 3.5–3.9 ppm (PEG ether protons, 1817H, multiplet)).

2.1.3. 4-Arm PEG-Norbornene synthesis

4-arm 20 kDa PEG-OH and 4-arm 20 kDa PEG-NH_2 were functionalized with norbornene (forming PEG-ester-norbornene or PEG-amide-norbornene) using N,N′-dicyclohexylcarbodiimide (DCC) coupling as previously described [28,42]. Norbornene carboxylate (10 meq per PEG arm), DCC (5 meq), pyridine (1 meq), and 4-dimethylaminopyridine (DMAP) (0.5 meq) were dissolved in 100 mL DCM for ~30 min at room temperature, and 5 g of 4-arm PEG dissolved in ~50 mL DCM was added dropwise. The solution was stirred at room temperature overnight and vacuum filtered. The filtrate was precipitated in 1 L ice-cold diethyl ether. The precipitate was collected by vacuum filtration, twice dissolved in 75 mL DCM, and precipitated in ice-cold diethyl ether. Structure and percent functionalization (>90%) were determined by ^{1}H-NMR: 4-arm PEG-ester-norbornene and 4-arm PEG-amine-norbornene (^{1}H NMR ($CDCl_3$): δ = 6.0–6.3 ppm (norbornene vinyl protons, 8H, multiplet), 3.5–3.9 ppm (PEG ether protons, 1817H, multiplet)).