Review Article

In vitro modelling of the mucosa of the oesophagus and upper digestive tract: narrative review

Kyle Stanforth[1], Peter Chater[1], Iain Brownlee[2], Matthew Wilcox[1], Chris Ward[1], Jeffrey Pearson[1]

[1]NUBI, Newcastle University, Newcastle upon Tyne, UK; [2]Applied Sciences (Department), Northumbria University, Newcastle upon Tyne, UK

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Correspondence to: Kyle Stanforth. NUBI, Medical School, Framlington Place, Newcastle University, NE2 4HH, Newcastle upon Tyne, UK. Email: kyle.stanforth@ncl.ac.uk.

Abstract: This review discusses the utility and limitations of model gut systems in accurately modelling the mucosa of the digestive tract from both an anatomical and functional perspective, with a particular focus on the oesophagus and the upper digestive tract, and what this means for effective *in vitro* modelling of oesophageal pathology. Disorders of the oesophagus include heartburn, dysphagia, eosinophilic oesophagitis, achalasia, oesophageal spasm and gastroesophageal reflux disease. 3D *in vitro* models of the oesophagus, such as organotypic 3D culture and spheroid culture, have been shown to be effective tools for investigating oesophageal pathology. However, these models are not integrated with modelling of the upper digestive tract—presenting an opportunity for future development. Reflux of upper gastrointestinal contents is a major contributor to oesophageal pathologies like gastroesophageal reflux disease and Barratt's oesophagus, and *in vitro* models are essential for understanding their mechanisms and developing solutions. The limitations of current model gut systems in modelling the mucosa is not only limited to the oesophagus. Integration of modelling of the mucus covered epithelia of the stomach and small intestine in to upper digestive tract models is limited and often not considered at all. In this paper we discuss mucus structure and function and current approaches to modelling of the mucus layer in isolation, and in integrated systems with cell culture systems and digestive models. We identify a need for relevant modelling of the viscoelastic properties of mucus and its protective function to allow complete integration in modelling. Addressing limitations of current *in vitro* models and integrating upper gastrointestinal models with those of the oesophagus presents an opportunity for better understanding oesophageal physiology and pathophysiology where reflux of digestive fluids is involved.

Keywords: Oesophagus; model gut systems; mucus; organoids; *in vitro* models

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Introduction

The human 'swallowing apparatus' consists of the pharynx, the upper oesophageal sphincter (UOS), the oesophagus, and the lower oesophageal sphincter (LOS). Major functions of the oesophagus are to co-ordinate the movement of food from the mouth to the stomach, and to control the retrograde movement of digestive material and digestive secretions—which can become pathological if excessive (1). While ingested food transits through the oesophagus in a relatively short period of time compared to distal parts of the gastrointestinal tract (GIT), oesophageal dysfunction appears to result in negative consequence on both health and quality of life. Disorders of the oesophagus include heartburn, dysphagia, eosinophilic oesophagitis, achalasia, oesophageal spasm, gastroesophageal reflux disease (2).

Several methodologies for *in vitro* simulation of the digestive tract are available to researchers, however, few of these consider the role of the oesophagus. This is part of a wider limitation of model gut systems in accurately modelling the digestive mucosa from both an anatomical and functional perspective.

Current model gut systems have been reviewed extensively elsewhere with reviews variously describing *in vitro* models by:

- Type; static models (3), dynamic models (4), cell based models (5-7), *ex vivo* models (8);
- Functionality; models of microbiome (9), physiological relevance (10,11);
- Or application; food digestion (12), drug delivery (13), screening and toxicity (14), assessment of pre- and pro-biotics (15), protein stability (16), nutraceutics (17).

These systems have many benefits including controllability, repeatability, ethics, timing and cost (7), but known limitations include modelling of hormonal control, simulation of physical forces ["the topography, motility and flow" (7)], immune function of the digestive tract, and accurate modelling of the absorptive mucosa.

Accurate modelling of the mucosa is an aspect of gastrointestinal physiology absent from the vast majority of these models and has only been the focus of limited discussion in the literature. Here, in a review of current literature, we aim to address this notable omission, discussing models of the oesophageal mucosa and the digestive and absorptive mucosa of the upper GIT including mucus function, and integration of mucus modelling in to *in vitro* digestion and absorption systems.

We present the following article in accordance with the Narrative Review reporting checklist (available at https://aoe.amegroups.com/article/view/10.21037/aoe-20-90/rc).

Static and dynamic digestion models

In vitro digestion models aim to simulate the processes of digestion and absorption carried out by the organs of the GIT (*Table 1*). Static digestion models have been reviewed extensively by Alegria *et al.* (3), and in an effort to harmonise the various static methodologies, the COST INFOGEST partnership developed a consensus method for static digestion modelling with a view to facilitating comparison of data and repeatability (18).

Static models simulate luminal aspects of digestion in a stepwise manner, modelling the chemical and enzymatic environment of successive organs of the digestive tract (summarised in *Tables 2-4*) without taking into account some of the more complex physiological processes of the digestive tract that dynamic models aim to replicate including; secretion, gradual pH changes, physical forces of mixing, shearing and motility, gastric emptying, mucus permeation, absorption, and hormonal control.

The functionalities of certain dynamic gastrointestinal models are summarised here, in brief. The Dynamic Gastric Model (DGM) (48), Human Gastric Simulator (HGS) (49) and the Human Gastric Digestion Simulator (HGDS) (50) are computer-controlled mono-compartmental systems (MoCS) that aim to recreate gastric secretions, churning and emptying.

The DGM consists of a flexible main body surrounded by a water jacket, a piston and barrel recreate peristaltic forces by fluctuating the water pressure in the jacket to simulate contractions in the fundus and antrum (48). The HGS, is a latex vessel subject to simulated gastric forces from mechanical, belt-driven rollers (49). Data from the DGM and HGS (51-53), is reported to correlate well with *in vivo* data and the systems have been used for modelling transit and emptying times, gastric enzymatic digestion, particle breakdown, rupture times of delivery systems, phase separation, gel formation and nutrient/drug release (48).

The TNO Gastro-Intestinal Model (TIM-1), The DIDGI MGS, and the Engineered Stomach and small INtestinal MGS (ESIN) are multi-compartmental models of the upper GIT. The TNO Gastro-Intestinal Model (TIM-1) consists of four compartments simulating: the stomach, duodenum, jejunum and ileum (54-57). The DIDGI MGS (58) simulates the upper GIT with a 2-compartment system, representing the stomach and small-intestine—both stirred glass vessels surrounded by a heated water jacket. The pH changes, secretion rates and emptying times are computer controlled, based on data collected from *in vivo* experiments. The ESIN (59) is comprised of 6 compartments, including: a meal reservoir (R1) which allows continuous passage of food material into the stomach compartment, a salivary ampoule (R2) which mixes the food components with salivary enzymes and buffers, the stomach (R3), the duodenum (R4), the jejunum (R5) and the ileum (R6).

While some of these systems have the capacity to model the oral phase and/or salivary secretions, none of them model oesophageal function. This reflects the limited role the oesophagus is considered to have in digestion, acting as a transport corridor between the mouth and stomach. However, modelling oesophageal function and interaction

Table 1 Organs of the digestive tract, their respective sub-compartments, and functions

Organ	Function
Mouth (oral phase)	Breaks down foods into small particles via mastication
	Lubrication of food for bolus formation and swallowing via saliva
	Digestive enzymes in saliva (amylase and lingual lipase) begin digestion
Oesophagus	Transport of ingested material from the mouth to the stomach
	Sphincters in the lower and upper oesophagus prevent reflux of stomach contents into the mouth and airways, while still allowing regurgitation and vomiting when required
Stomach (gastric phase)	Storage of food
	Chemical digestion via hydrochloric acid and pepsin
	Mechanical digestion via churning
	Pathogen defence via strong acid, and pepsin
Duodenum (SI phase)	Neutralises stomach acids with bicarbonate secretions
	Mixes chyme with bile from the liver for lipid digestion
	Mixes chyme with pancreatic enzymes for carbohydrate, protein, and lipid digestion
Jejunum (SI phase)	Bulk absorption of simple carbohydrates, peptides, amino acids, and fatty acids
Ileum (SI phase)	Absorbs nutrients not taken up by the jejunum, e.g., vitamin B_{12}
	Also important for uptake and redistribution of bile salts and remaining water-soluble vitamins
Large intestine (colonic phase)	Reabsorbing water and vitamins not absorbed by small intestine
	Housing the large intestinal microbiome
	Absorbing SCFAs produced by the large intestinal microbiome
	Storage of faeces

Table 2 Digestive secretions of the gastrointestinal tract

Region	Name	Secretion	pH optima	Function
Mouth	Lingual lipase	Von Ebner glands (19)	4.5–5.5 (19)	Digestion of triglyceride aggregates via ester bond hydrolysis, digestion continues until material reaches the stomach (19)
	Salivary amylase	Parotid gland (20)	5.6–6.9 (21)	Digestion of insoluble starches into progressively smaller soluble starches, with smallest being maltose, via hydrolysis of 1,4 glycosidic bonds (20)
Stomach	Pepsin	As pepsinogen from chief cells (22)	2 (22,23)	Pepsinogen autolysed into pepsin. Pepsin digests proteins via hydrolysis of peptide bonds. Pepsis cleaves at the C-terminal of hydrophobic, preferentially aromatic, i.e., phenylalanine, tyrosine and tryptophan, residues (22,23)
	Gastric lipase	Chief cells (24)	4.5–5.5 (25)	Similar to lingual lipase, triglycerides are digested via hydrolysis of ester bonds linking fatty acids to glycerol (25)
	Hydrochloric acid	Parietal cells (22)	–	Gastric HCl converts pepsinogen into pepsin rendering it an active protease, the acid also acts corrosively on proteins causing them to denature, exposing the peptide bonds to pepsin (12)

Table 2 (*continued*)

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Region	Name	Secretion	pH optima	Function
Small intestine	Trypsin	Acinar cells as trypsinogen (26)	7.0–8.0 (26)	Trypsinogen, the trypsin zymogen, is activated by enterokinase and/or trypsin via cleavage in the duodenum. Active trypsin then cleaves peptide bonds on the carboxy side of lysine and arginine (26)
	Chymotrypsin	Acinar cells as chymotrypsinogen (27)	7.0–8.0 (27)	Chymotrypsinogen, the chymotrypsin zymogen, is activated via cleavage by trypsin and autolysis. The active enzyme hydrolyses peptide bonds at the n-terminal side of aromatic amino acid residues (27)
	Carboxypeptidase A	Acinar cells as procarboxypeptidase (28)	~7 (29)	Secreted as procarboxypeptidase and activated via trypsin cleavage, carboxypeptidase is an exopeptidase that cleaves peptide bonds of peptides with amino acids with free COOH groups giving a single amino acid as product. Carboxypeptidase A does not cleave peptide bonds when the relevant amino acid is proline, lysine, arginine or histidine (28)
	Elastases	Acinar cells (30)	8.2–9.2 (31)	Elastases are serine proteases which cleave peptide bonds on the c-terminal side of hydrophobic amino acids (30)
	Pancreatic lipase	Acinar cells (32)	8 (32)	Pancreatic lipase, following binding of its cofactor—colipase, hydrolyses ester bonds of triglycerides at the oil-water interface after the emulsification of lipids by bile. The resulting product is 2 free fatty acids and a monoacylglycerol (32)
	Phospholipases	Acinar cells (33)	6–8 (34)	Phospholipase enzymes act by hydrolysing the ester bonds of phospholipids, releasing a fatty acid (33)
	Pancreatic amylase	Acinar cells (35)	7 (36)	Like salivary amylase, pancreatic amylase hydrolyses 1,4 glycosidic bonds in oligosaccharides, resulting in maltose, maltotriose and limit dextrins (37)

Table 3 Brush border enzymes present in small intestinal enterocytes

Class	Name	Function
Carbohydrate	Sucrase-isomaltase	Sucrase isomaltase functions as carbohydrase dimer of sucrase and isomaltase. The sucrase subunit hydrolyses the glycosidic bond in sucrose to release glucose and fructose. The isomaltase subunit hydrolyses 1,6 glycosidic bonds in limit dextrins (38)
	Lactase	Hydrolyses the β-1,4 glycosidic bonds in lactose, found in milk and dairy products, into glucose and galactose (38)
	Maltase-glucoamylase	Composed of two subunits, maltase and glucoamylase, which with differing substrate specificity hydrolyse α-1,4 glycosidic bonds at the non-reducing side of oligosaccharides in the gut lumen, releasing terminal glucose (38)
	Trehalase	Hydrolyses the 1,1 glycosidic bonds found in trehalose, releasing glucose (38)
Proteins	Peptidases	There is a vast array of peptidases found in the brush border of the small intestine, and these enzymes display varying degrees of substrate specificity, i.e., target amino acids and length of peptide, these specificities have been previously discussed (39)
Lipids	Phospholipase	Phospholipase A2 has been noted to be present in the brush border of the small intestine, where it will hydrolyse bonds which link fatty acids to phospholipids (40)

Table 4 Important gastrointestinal regulatory hormones

Name	Secretion	Function
Cholecystokinin (CCK)	Duodenal enteroendocrine cells (41)	While Known for a role in satiety, CCK also stimulates the secretion of pancreatic enzymes and hormones and stimulates motility of the gastrointestinal tract. CCK secretion is stimulated by the presence of partially digested proteins and gastric acid in the duodenum (42)
Gastrin	G cells (43)	Gastrin, secreted from G cells in the stomach and duodenum, stimulates secretion of gastric acid, as well as pepsinogen and intrinsic factor from parietal cells, and secretin (42) from S cells (44)
Secretin	S cells (44)	Secretin, secreted by S cells in the duodenum, inhibits the release of gastrin and gastric acid, lowers pressure of the lower oesophageal sphincter, and stimulates pancreatic release of bicarbonates, digestive enzymes and insulin (44)
Vasoactive intestinal polypeptide (VIP)	Neurons and immune cells (45)	Secretion of water and electrolytes into the duodenum following the secretion of pancreatic juices and bile. Also inhibits gastric acid secretion and relaxes smooth muscles of the gut (42)
Gastric inhibitory peptide (GIP)	SI K cells (46)	Secreted primarily in the duodenum and upper jejunum GIP inhibits gastric secretions and stimulates secretion of somatostatin which intensifies the downregulation of gastric secretions (47) However, the most important role of GIP is the potent stimulation of insulin release (42)
Other peptides	Vary	There are other hormones and peptides that have an influence on gastrointestinal regulation, albeit to a lesser extent than those above. These include somatostatin which downregulates gastrointestinal processes, ghrelin which stimulates gastric secretions during hunger, motilin which stimulates gastrointestinal motility and many others (42)

with the oral secretions and refluxed material from the upper digestive tract could bring important understanding of oesophageal pathologies.

Physiology of the oesophagus

The oesophagus is a 25–30 cm long, hollow, muscular tube, which connects the oral cavity and pharynx to the stomach (60). The oesophagus is split into two sections, the upper cervical section which makes up the first 2–6 cm of the oesophagus, and lower thoracic section (61). The UOS forms the junction between the pharynx and the cervical oesophageal region, and the LOS forms the junction between the thoracic oesophageal region and the stomach (60). The oesophagus transports ingested material, often in the form of a food bolus, from the oral cavity to the stomach via peristaltic waves which propagate down the oesophagus, shifting the bolus towards the stomach (62). A secondary function is to prevent reflux of gastric contents back into the oral cavity and to allow regurgitation and vomiting—a function mediated by the oesophageal sphincters (62).

Tissue structure

The oesophageal tissue is comprised of 4 layers: the mucosa, the submucosa, the muscularis externa and the adventitia (63). The mucosa is the uppermost layer on the luminal side of the oesophagus. It is composed of a surface epithelium (*Figure 1A*), a lamina propria and muscularis mucosae (*Figure 1B*). The oesophageal surface epithelium is a non-keratinised, stratified, squamous epithelia of around 30 cell layers (63-65). These cells are present throughout the oesophagus until a small area surrounding the LOS where gastrointestinal-like columnar cells may be observed (63). In Barrett's Oesophagus, squamous epithelial cells around the LOS are replaced with columnar epithelial cells due to metaplasia (69). Found in the lower mucosal layers, below the basement membrane, is the lamina propria—a loose connective tissue rich with immune cells (63)—and the muscularis mucosae, a thin layer of longitudinal muscle cells (70). The mucosa is the primary defence against underlying tissue damage from acid refluxate (i.e., hydrochloric acid and pepsin), generating both pre-epithelial and epithelial defensive actions, most of which are provided by the specialised epithelium (65).

The submucosa (*Figure 1B*) is comprised of connective tissue, lymphocytes, plasma cells, the submucosal plexus and submucosal glands (63) which secrete bicarbonate ions and mucin (71). Bicarbonate ions function to neutralise acids during reflux of gastric contents into the oesophagus (71).

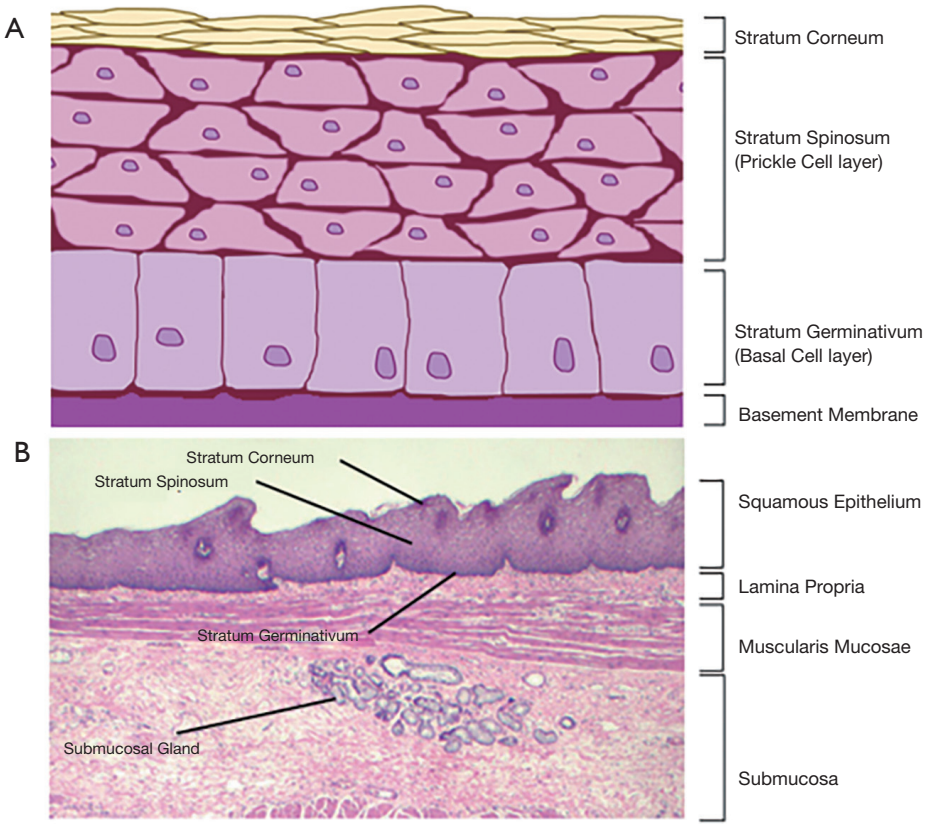

Figure 1 Diagrammatic representation of oesophageal tissue structure. (A) A diagram of the squamous epithelial layer of the oesophageal mucosa. The upper cell layer, the stratum corneum, is 7–8 cell layers of cornified epithelial cells which functions as a protective barrier against acid and abrasion (64). Below this is the stratum spinosum, an intermediate cells layer where squamous cells mature before undergoing cornification close to the luminal surface to form the stratum corneum (65,66). Attached to the basement membrane is the stratum germinativum, or the basal layer, containing the cells which undergo mitosis to replenish the upper oesophageal layers (67). (B) A histological section of the oesophageal mucosa and submucosa, adapted from Tracht *et al.* [2020] (68). In the upper squamous epithelial layer the stratum corneum, stratum spinosum and stratum germinativum can be seen. Below lies the lamina propria, the muscularis mucosae and the submucosa. The submucosa here can be seen housing a submucosal gland, which secretes mucin and bicarbonates.

Secreted mucin, MUC5B specifically in this case (72,73), typically forms surface mucus (74-76). In the oesophagus however, it has been noted that the levels of MUC5B secretion by submucosal glands are not sufficient to provide a fully functioning surface mucus layer (hence the absence of a mucus layer in the oesophagus) (77), thus the mucin secretions most likely function to help lubricate the bolus as it passes through the oesophagus.

The muscularis propria is important for the generation of peristaltic waves which propagate boluses down the oesophagus. It consists of up to 33% striated muscle in the cervical region and transitions to smooth muscle in the lower thoracic region. There is a transition region as the striated muscle begins to change to smooth muscle, this is identified by a mixture of striated and smooth muscle cells and a lack of significant contractions (62,63). Interestingly, striated muscle of the upper cervical region is not controlled voluntarily despite being innervated with somatic motor neurons, instead being controlled by several reflex inputs which bring about peristaltic contractions. On the contrary, smooth muscle present in the thoracic region of the oesophagus creates peristaltic waves via stimulation of the myenteric plexus (61).

Finally, and unlike the remainder of the GIT, the oesophagus does not contain a serosa layer, instead having an adventitia, a base layer of fixed connective tissues, binding the oesophagus to the surrounding tissue and holding it in place (63).

The oesophageal sphincters

The UOS sphincter is the musculo-cartilaginous high-pressure zone at the end of the pharynx and start of the cervical region of the oesophagus. This anatomical sphincter serves as the first barrier to the oesophagus. It prevents both the reflux of gastric contents into the oral cavity, of which hydrolytic enzymes and acid would cause damage to tissues, and the flow of inhaled air from entering the digestive tract, while also allowing vomiting and regurgitation in the necessary scenarios. Like the rest of the cervical portion of the oesophagus, the muscles surrounding the UOS are striated, skeletal muscles. Moreover, it is similarly controlled involuntarily, despite being under the influence of somatic nerves, being controlled rather on the basis of a variety of reflex inputs to the innervated motor neurons (70,78).

The LOS sphincter is not easily identified as the characteristic thickening of muscle tissue common to anatomical sphincters is not seen with the LOS as functionality occurs through maintenance of muscle tone, modulated by several excitatory and inhibitory vagal pathways in the muscles modulating sphincter function (70,79-83). The functionality is controlled by two muscles; the smooth muscle of the lower thoracic oesophagus, and striated muscles fibres found in the crural diaphragm (82,84). The smooth muscle is organised in C-shapes, rather than rings, which interact with gastric sling fibres. When in a contracted state, the smooth muscles clasp together and pull in the gastric sling fibres, creating a closure, and during relaxation, in the instance of passing food for example, the clasp is released to create an opening (85). The striated diaphragmatic sphincter muscles work in tandem with the smooth muscle providing functional support (81,85).

Oesophageal defence and associated disease

The surface mucus gel coating the epithelium and secreted bicarbonates form a pre-epithelial defence in the majority of the GIT (86,87). For example, mucin glycoproteins in gel format provide an exclusion barrier in the small intestine by forming pores around 200 nm in size, providing protection against larger toxic particles, enzymes and microorganisms (88). Moreover, mucus in the stomach and small intestine protects the epithelia from enzymatic degradation (86), while facilitating the production of an unstirred water layer, rich in secreted bicarbonate ions to neutralise the harsh acid present in the stomach and duodenum (87). Mucus layers in the GIT, particularly those formed in the intestine by MUC2, are also known to produce an exceptionally rigid, firm mucus layer close to the epithelia which is often sterile even in the colon—a region where bacterial counts are $\times 10^{10}$–$\times 10^{12}$ per gram faecal matter—implicating its function as a mucosal protectant from microorganisms.

Though the oesophagus is associated with secreted mucus, with oesophageal submucosal glands strongly expressing and secreting mucin MUC5B (73), oesophageal epithelia scrapings have shown that the secretion is not significant enough to form a functioning surface mucus layer (0.47 $\mu g/cm^{-2}$ oesophageal scrapings *vs.* 500 $\mu g/cm^{-2}$ of stomach scrapings) (77). In the healthy oesophagus, bicarbonate secretions and salivary bicarbonates, are enough to limit epithelial damage to the oesophagus by acids (77). However, when acid levels are higher than normal during a reflux event the oesophagus is vulnerable to damage without a protective bicarbonate rich mucus layer (87).

Like the epithelium of the oral cavity, stratified squamous epithelium protects the delicate tissues below from abrasion, but the stratified squamous epithelia of the oesophagus have other specialised mechanisms that allow it to cope with acid reflux. The apical membrane has been shown to prevent H+ permeation into cells despite the presence of non-selective cation channels—it is thought that low pH causes a reduced influx of cations (39). Moreover, the apical junction complex, a complex which fuses adjacent cells in the stratum corneum and spinosum, regulates paracellular diffusion of H+ ions. There are 3 structures in total which make up the complex: closest to the apical side of the membrane are the tight junctions, this protein structure spans over the membranes of two cells and interlinks their cytoskeletons, bringing the membranes tight to one another. This complex is the main paracellular transport modulator of the complex. Found below the tight junctions are the adherens junction and found further below, close to the basolateral membrane, are desmosomes. Both protein structures provide structural support for the tight junction, while also having other regulatory roles within the cell. It is worth noting however that MUC1, a transmembrane mucin expressed in the surface epithelia of the oesophagus (73), may provide some protective functions, as studies have shown that MUC1 contains Lewis antigens—a feature of the epithelial glycocalyx where *H. pylori* is known to occur. In this circumstance, the MUC1 Lewis antigens

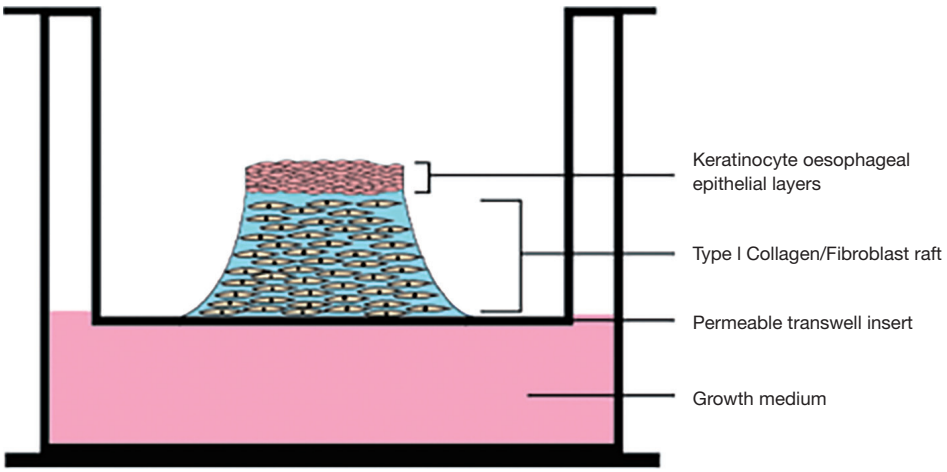

Figure 2 An oesophageal organotypic 3D culture system. The system depicts the end point of the experiment when the keratinocyte epithelial layer, sitting atop a collagen/fibroblast raft, has matured and is ready for analysis (96).

act as a decoy by binding the *H. pylori*—this is followed by a complete release of MUC1 from the membrane, with MUC1 establishing its protective role by carrying the infective material far from the epithelia (89,90).

Despite these defence mechanisms, they are often easily overcome by unusually high levels of gastric reflux of acid and enzymes. High levels of reflux are often associated with disease, and depending on the severity of the cause, they can cause varying degrees of damage to the oesophageal mucosa, from minor issues, like tissue damage from acids, to serious issues like Barratt's oesophagus and oesophageal adenocarcinoma (OADC).

In vitro oesophagus models

In vivo and *ex vivo* animal models, typically rodents like mice and rats, have been used to investigate oesophageal physiology (91,92). However, substantial differences between human and mouse oesophagus are a limitation. For example, the rodent oesophageal epithelia consists of around 4–6 layers, whereas that of a human consists of much thicker 25–30 layers and contains papillae like structures (91,92). Moreover, the mucosal layers of rodents are keratinised, as opposed to the non-keratinised cells present in humans, and do not contain mucus secreting glands (93). Oesophageal human cell models are widely used today (94) and continue to advance in complexity—with poor *in vivo* correlation of results from 2D monolayer methods leading to a focus on developing 3D models that accurately recapitulate the architecture and functionality of oesophageal tissue (95).

Organotypic 3D Raft Culture (OTC)

Transwell based Organotypic 3D Raft Culture (OTC) models utilise a transwell system in which submerged fibroblast cells, grafted onto a collagen matrix on the surface of the apical transwell membrane, act as a platform or "raft" for keratinocytes to differentiate into a functioning epithelial layer (*Figure 2*) (96,97). This model has been applied to a variety of tissues and have been used widely to investigate tissue development, disease and also for toxicity assays (97). OTC has been successfully used to model oesophageal epithelial generation and proliferation (98,99), oesophageal squamous cell carcinoma (ESCC) (100-102), eosinophilic oesophagitis (94) and Barratt's oesophagus associated OADC (103-105).

For example, in the work of Kalabis *et al.* [2012] (96), oesophageal fibroblasts were embedded in bovine collagen type I on an insert on top of a collagen matrix coated membrane and cultured for 7 days, forming a collagen/fibroblast raft on the insert membrane. Oesophageal keratinocytes were then seeded on the surface of the matrix and allowed to develop over the course of 4 days. Keratinocytes present at the air/liquid interface promote stratification and differentiation in the epithelial layer, generating epithelial layers representative of the oesophageal mucosa, recreating the stratum corneum, stratum spinosum and stratum germinativum, though the underlying tissue structures—which remain important to

oesophageal function—are not recreated using this system.

3D multicellular spheroid culture and sphere formation assays

Multicellular spheroid culture, the first of the submerged 3D models used in oesophageal research, allows dissociated epithelial cells to reaggregate under submerged conditions. This reaggregation gives rise to self-organised spheres which resemble *in vivo* oesophageal tissue organisation (106). There are a variety of multicellular spheroid culture models (spinner flask suspension culture, liquid overlay culture, hanging drop culture and microfluidic culture), all with their own advantages and disadvantages, these are detailed in the review by Mehta *et al.* [2012] (107).

Multicellular spheroid culture have been shown to be effective models in drug permeation (107), cell functionality (108), tumour angiogenesis (109), and tumour-immune cell cross talk (110). In the oesophagus specifically, these models have been used to study ESCC-cell interactions (111), sphere formation in OSC-1 and 2 cell lines (98,112) and comparisons of spheroid formation between cell types (113). The associated limitations are the maintenance of uniform spheres, variability in cell type numbers with each sphere, and a lack of standardised methods for drug screening (114).

Sphere formation assays, another type of spheroid model, follow a similar principle to multicellular spheroid culture, but rather than allowing dissociated cells to aggregate and self-organise, multipotent stem cells from tissues are prevented from aggregating, allowing them to propagate and differentiate into single cell derived spheroids (115). This method has allowed research into the proliferation, self-renewal and multipotency of EADC (116,117) and ESCC (118-120) stem cells, which are known for their influence in their respective cancers.

3D organoids

3D organoids build on the ideas of the previous 3D culture models, aiming to accurately replicate *in vivo* organ architecture, processes, and parameters on a miniature scale. This is typically done using a single or small number of induced pluripotent or embryonic stem cells, embedded in an extracellular matrix hydrogel to support growth (often Matrigel) (121,122). These multipotent cells are allowed to differentiate, propagate and self-organise into a miniature organ-like scaffold containing a vast array of functional cell layer and types (122,123). The vast array of cell lineages and types give an accurate representation of organ parameters and architecture, unlike that of spheroid culture and OTC. Moreover, 3D organoids are much easier to maintain due to their ability to self-renew. In 3D organoid culture, a patient's own stem cells can be used, which may have significant implications in the future of personalised medicine (122). Research using oesophageal organoids includes development, differentiation/proliferation and therapeutic approaches to EADC (124,125) and ESCC (126-128), oesophageal signalling pathways in GORD (94,129) and eosinophilic oesophagitis (94).

Absorption modelling—epithelial absorption and mucus function

Similarly, there are limitations to modelling of the intestinal digestive mucosa. Nutrient and drug absorption from the digestive tract involves mucus permeation, brush border enzyme activity and transport across the epithelia via passive diffusion, carrier mediated diffusion, active transport, and pinocytosis (*Table 5*) (135). Brush border enzymes and specific transport mechanisms are summarised in *Tables 2-4*. The use of *in vitro* cell models to simulate intestinal absorption is common, but systems such as the Caco-2 monolayer model do not include a mucus layer (136), other approaches are discussed later.

Mucus layers coat the epithelia and protect from microbial and enzymatic damage, while also allowing absorption of nutrients. The presence of a mucus layer does however, limit the absorption of certain nanoparticles and drugs *in vivo* (88). With mucus being a limiting factor for absorption *in vivo*, it is essential that the mucus layer is accounted for in absorption models.

Mucus is a negatively charged hydrogel, and is typically ~95% water with the glycoprotein mucin (*Table 6*) being the gel forming component (88,139,140). Mucins are categorised into either 2 subgroups, membrane tethered and secreted (139,141-143). Mucins are high molecular weight glycoproteins varying in size, domain organisation and function—despite this, there are common structural features. The protein backbone of the mucin glycoproteins consists of serine, threonine and proline (STP) repeats. These STP repeats are the fingerprint of mucin molecules. Each mucin type has a unique STP repeat sequence known as a variable number tandem repeat (VNTR) region (88,139,142,144). These are important regions which function as the site of carbohydrate chain attachment,

Table 5 Small intestinal epithelial absorption and efflux mechanisms

Class	Mechanism	Function
Carbohydrate	SGLT1	Na+ dependent uptake of glucose and galactose from the small intestine. Uptake is mediated by a Na+ concentration gradient maintained by an Na+/K+ ATPase pump which creates a high concentration of Na+ ions in the small intestinal lumen, this allows glucose and galactose symport across the membrane. This is an example of secondary active transport (130)
	GLUT5	Facilitated diffusion of fructose from a high concentration on the small intestine to a low concentration in the enterocytes (130)
	GLUT2	Basolateral facilitated diffusion of glucose, galactose, and fructose into serum (131)
Peptides	PEPT1	H+ dependent symport of di and tripeptides into enterocytes from the small intestinal lumen, peptides are then hydrolysed to amino acids in the cells by internal peptidases (130)
Amino acids	ASC	Na+ dependent antiport uptake of alanine, serine, cystine, threonine, glutamine and asparagine (132)
	B0	Na+ dependent symport uptake of neutral L-amino acids (130,132)
	B0,+	Na+ and Cl- dependent symport of neutral and cationic amino acids as well as β-ala (130,132)
	b0,+	Independent uptake of cysteine, neutral amino acids and cationic amino acids (130)
	β	Apical and basolateral Na+ and Cl-dependent transport (132) of taurine and β-alanine (130,132)
	Gly	Na+ and Cl-dependent basolateral symport of glycine into serum from enterocytes (132)
	IMINO	Na+ and Cl-dependent symport uptake of proline, hydroxyproline (132) and pipercholic acid (130)
	L	Basolateral transport of neutral amino acids (except proline) from enterocytes into serum (132)
	N	Na+ (symport) and H+ (antiport) (132) dependent uptake of glutamine, asparagine and histidine (130) bro
	PAT	H+ dependent symport uptake of proline, glycine and alanine (130,132)
	T	Facilitated diffusion uptake of aromatic amino acids phenylalanine, tyrosine and tryptophan (132)
	X-AG	Na+, H+ (symport) and K+(antiport) dependent uptake of (anionic) aspartate and glutamate (132)
	Y+L	Na+ dependent cotransport uptake with neutral amino acids (132)
Lipids	CD36	Facilitates fatty acid uptake from the small intestinal lumen (133)
	NPC1L1	Cholesterol uptake transporter (130)
	ABCG5, ABCG8	Efflux of cholesterol from enterocytes into small intestinal lumen (130)
Vitamins	SVCT1, SVCT2	Na+ dependent vitamin C uptake via Na+ cotransport (130)
	SMVT	Na+ dependent uptake of biotin by enterocytes (130)
	Cubam	Not a transporter as such, but a receptor composed of amnionless and cubulin, responsible for binding the intrinsic factor/vitamin b12 complex, allowing endosome uptake (134)
	FOLT, PCFT/ HCP1, FOLR1	Small intestinal folate uptake occurs through these three transporter proteins (130)
	RFVT1, RFVTR	Small intestinal uptake of riboflavin is thought to occur using these two transporters (130)
	THTR1, THTR2	Small intestinal thiamine uptake occurs using these two transporters (130)
	Other vitamins	Fat soluble vitamins, A, D, K and E typically follow the same route as lipids, which are organised into micelles and absorbed (130)

Table 6 Other secretions critical for gastrointestinal function

Name	Function
Mucin	Mucins are heavily glycosylated glycoproteins which are the major component of secreted mucus, a heterogeneous hydrogel which protects the epithelial layers of the gastrointestinal tract from chemical, mechanical, and pathogenic damage, while also providing an essential nutrient source for intestinal flora. Mucins also come in membrane tethered forms which have roles in epithelial protection, the immune response and cell signalling (88)
Intrinsic Factor	Secreted by gastric parietal cells, intrinsic factor is a glycoprotein which is essential for the absorption of cobalamin (vitamin B12). Intrinsic factor is present in the small intestine when cobalamin is release from its protein complex and becomes available for binding. The cobalamin/intrinsic factor complex binds to receptors on the epithelial cells of the terminal ileum, prompting absorption (137)
Bile	Bile, conjugated in the liver (as bile acids) and stored in the gall bladder, are an essential component of lipid absorption. Lipids are not water soluble; this makes the surface area for enzymatic action low. Bile works by emulsifying lipids, creating an oil: water interface at which pancreatic lipase and its cofactor, colipase, allows effective digestions of lipids into their simple forms, where they can be absorbed (138)

primarily by O-glycosylation, which make up ~70% of the mucin molecular weight (145). The presence of carbohydrate chains on secreted mucins gives them the ability to form gels (146). O-glycosylation occurs when N-acetyl galactosamine (GalNAc) residues are covalently linked to serine or threonine by glucotransferases via an OH group (147). A progressive elongation of the carbohydrate chain, by Golgi glucosyltransferases, up to 20 residues in length occurs using four other carbohydrate residues: N-acetyl glucosamine, fucose, galactose and sialic acid (88,139,144). Proline's role in the VNTR region is thought to be assistance in the linkage of GalNac residues to serine and threonine (147). Statistical analysis has noted a much higher number of proline residues surrounding glycosylated serine and threonine residues when compared to their non-glycosylated counterparts (148). with affinities depending on proline's proximity to the O-glycosylation site, with proline at +3 and –1 strongly favouring O-glycosylation as opposed to proline at other sites (149).

Carbohydrate side chains are heavily sulphated upon elongation—it is their presence, alongside sialic acid, which allow mucus to have a negative charge at physiological pH levels (88).

Secreted mucins (*Figure 3; Table* 7), provide a mucus layer for several mucosal surfaces. Of these, MUC2, was one of the first secreted mucins to be characterised in detail (88,139,151-158). MUC2 (*Figure 3*) is the predominant secreted mucin of the SI (88,152,159,160) and the colon (152,157,158). At the N-terminal, vWF-like domains D1, D2, D' and D3 are found, while at the C-terminal a vWF-like D4 domain is found, followed by a B, C and CK domain. The central region of MUC2 is split into two VNTR regions, a short region closer to the N-terminal which is flanked by 2 globular cysteine domains (CYS) domains at either side, and a longer region which spans most of the backbone. These regions are separated by the single globular CYS domain found at the C-terminal side of the first VNTR region (88,151,157). The initial short VNTR region is conserved and shows little variability in the number of STP repeats which are generally 23 amino acids long (151). The longer VNTR region however is highly variable with 40–185 STP repeats (151,161) which contributes to its large size of up to 7 mDa (155).

The mechanism of secretion of MUC2 has been well characterised and is described in detail by Pearson *et al.* [2016] (88), Ambort *et al.* [2011; 2012] (153,154) and Johansson *et al.* [2011] (157). In the endoplasmic reticulum at pH 7.2, the MUC2 monomers are linked in trios at the D3 domain via disulphide bridges, creating MUC2 trimers. These trimers are transported to the Golgi Apparatus where the bulk of O-glycosylation takes place. In the Golgi Apparatus, the pH drops to 5.2—this causes protonation of histidine residues which in turn allows non covalent interactions between the vWF-like domains D1, D2 and D3, creating 5 or 6 sided ring-like structures which operate with D3 as the corners. An increase in Ca2+ levels in the Golgi Apparatus allows cross linking of negative charges, allowing the mucins to be tightly packed into secretory granules. A final dimerisation at the CK domain ensures that the mucin polymers are ready for secretion. Upon secretion, Na+ ions displace Ca2+ ions, uncoupling the cross links—this allows mucus to rapidly hydrate and swell 1,000–3,000-fold.

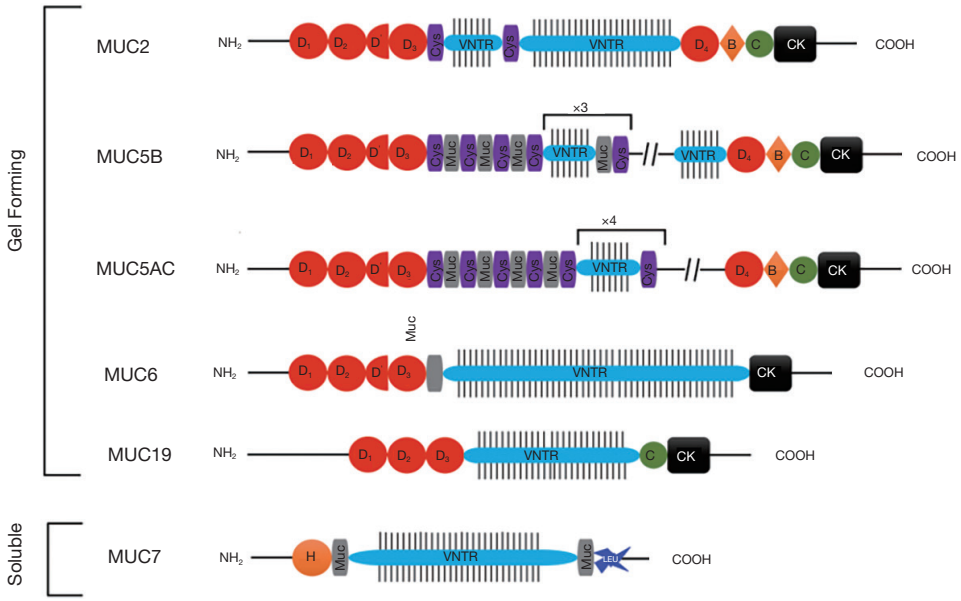

Figure 3 Diagrammatic representation of 5 secreted gel forming mucins (MUC2, MUC5AC, MUC5B, MUC6 and MUC19) and secreted soluble mucin MUC7. D represents vWF-like D domains (red). They are numbered D1-D4, with the first 3, as well as D' which represents half a D domain, are found at the N-termini of gel forming mucins. D4 is often found at the C-termini of gel forming mucins. Also found at the C-termini of gel forming mucins is the CK domain (black) which represents a cysteine knot, as well as B (orange) and C (green) domains similar to those in vWF—this exempts MUC6 which does not contain B, C or D4 domains. In the central region, all mucins contain some form(s) of heavily O-glycosylated VNTR region (blue) and may also contain non-repetitive mucin sequences (grey) and globular cys domains (purple). MUC19 is differs in structure from many of the other secreted gel forming mucins as it lacks a D', D4 and B domain, while also having an unusually long amino terminus. MUC7, the secreted soluble mucin, is much different from the other mucins and contains only a histidine domain (orange), a VNTR region flanked on either side by non-repetitive sequences, and a leucine zipper at the C-terminus (150,151).

MUC2 polymers are arranged into 5- or 6-sided ring like structures which act as pores. Research suggests these pores vary in size due to the sliding of the mucus layers but are understood to have a size limit of roughly 200 nm in diameter (88). The CYS domains are thought to determine their pore size through non-covalent bonds between half-cysteine residues and adjacent, positively charged amino acids (154). These pores allow particles smaller than 200 nm to traverse the mucus layer and size exclude larger particles. With no mucus layer, particles larger than 200 nm will easily access the epithelia. Moreover, the strong negative charges will cause mucus/particle interactions. Small neutral particles will likely cross the mucus layer with little hindrance. Negatively charged particles will likely be repelled by the mucus layers negatively charged residues, and positively charged particles will bind negatively charged residues and become trapped.

Approaches to mucus modelling

Groo *et al.* [2014] identified that although many methods for mucus diffusion studies are available there is not a standard consensus protocol (162). Several *in vitro* and *ex vivo* approaches have been adopted to mucus modelling, some of which are integrated with cell-culture systems, and some which are non-integrated and model the mucus layer independently.

Li *et al.* [2013] described the lack of mucus modelling as a limitation of Caco-2 monoculture systems, and although they do express membrane bound MUC1, they do not produce secreted intestinal mucins (136). As an advance on CACO-2 monoculture, co-culture of Caco-2 and mucus producing HT29 cells have been developed. HT29-MTX cells only secrete gastric type MUC5AC, whereas HT29-FU cells adapted with fluorouracil secrete intestinal type mucin MUC2 (163). While a key advantage of co-culture

Table 7 Mucin genes, their types, chromosomal location, and the tissues that express them [adapted from Pearson *et al.* 2016 (88) and McGuckin *et al.* 2011 (143)]

Mucin gene	Secreted/membrane	Tissue expression	Chromosomal location
MUC 1	Membrane	All epithelia, breast, pancreas, small intestine, bladder	1q21
MUC 2	Secreted (gel-forming)	Colon, small intestine, airways	11p15.5
MUC 3B	Membrane	Colon, small intestine, bladder	7q22
MUC 3A	Membrane	Colon, small intestine, bladder heart, liver, thymus, pancreas	7q22
MUC 4	Membrane	Airways, colon, small intestine, stomach, cervix, eye	3q29
MUC 5AC	Secreted (gel-forming)	Airways, stomach, cervix, middle ear, eye	11p15.5
MUC 5B	Secreted (gel-forming)	Airways, submaxillary gland, cervix, gallbladder, middle ear	11p15.5
MUC 6	Secreted (gel-forming)	Stomach, gall bladder, cervix	11p15.5
MUC 7	Secreted (soluble)	Salivary glands, airways, eye	4q13-21
MUC 8	Membrane	Airways	12q24.3
MUC 9	Membrane	Oviduct	1p13
MUC 12	Membrane	Colon	7q22
MUC 13	Membrane	GI tract, colon, airways	3q13.3
MUC 15	Membrane	Colon, airways, small intestine, spleen, prostate, breast, eye	11p14.3
MUC 16	Membrane	Ovarian epithelial cells, nasal mucosa, eye	19p13.3
MUC 17	Membrane	Duodenum, stomach, colon	7q22
MUC 18	Membrane	Lung, breast	11q23
MUC 19	Secreted (gel-forming)	Salivary glands, trachea submucosal glands	12q12
MUC 20	Membrane	Kidney placenta, colon, lung, eye, prostate, liver, eye	3q29
MUC 21	Membrane	Lung, colon, thymus	6p21.32
MUC 22	Membrane	Lung	6p21.31

systems is that the mucin is directly secreted above, and in intimate contact with the epithelia, the secreted mucin layer is not complete, and with a reported thickness of ~4 μm, which does not recapitulate an *in vivo* human mucus layer (which is two orders of magnitude thicker) (164). To allow for full integration of epithelial culture systems with digestive models, it is essential that the mucus layer retains relevant permeation characteristics, and can protect the underlying epithelia from components of digestive secretions. To our knowledge there is no published data on Caco-2-HT29-compatibility with digestive secretions, however it would be surprising if the thin mucin covering produced in co-culture would be sufficient to achieve either of these requirements. Despite limitations, the co-culture model is reported to generate more predictable experimental results compared to monoculture, and co-culture systems have been advanced further to integrate intestinal M-Like cells in a triple culture system (165).

Mucus/mucin source is one of the key considerations, and Lock *et al.* discuss the importance of factors including species, age, state of health, anatomical site of collection and inter-donor (animal or human) variability (163). Inter-donor variation can be somewhat overcome by pooling mucus from multiple donors prior to testing, and that samples can be stored frozen without significant changes to mucus rheology.

We have previously argued that while many studies have used mucins to simulate mucus function, it is important that these mucins replicate the native state (88). Sigma purified mucin is one of the most widely available commercial

sources of mucin, but due to proteolytic degradation during purification is not structurally comparable to native mucin, cannot form a gel, and affects permeation characteristics (88,163).

Attempts have been made to develop 'biosimilar' mucus using polyacrylic acid (carbopol), BSA and linoleic acid in combination with Sigma porcine mucin to make the viscoelastic properties and microstructure more comparable to native mucus. While lipid and protein components are normal components of the mucus gel, polyacrylic acid is not, and significant research has been conducted to show mucin-carbopol interactions can bring about dramatic changes to viscosity (166). Because of its mucus modifying properties and inhibition of pepsin hydrolysis of mucus, carbopol has been investigated as a muco-protective agent (88).

In order to better replicate mucus thickness, our group in collaboration, developed a transmembrane permeation system using native porcine small intestinal mucus in a transwell set up with a resulting mucus layer of a thickness of 929±115 µm (167). While this is thicker than data reported for human small intestinal mucus thickness, the thickness is within the same order of magnitude, and allowed for a higher throughput, reproducible method from which time-course data could be collected, and permeation data can be normalised to *in vivo* mucus thickness. This has advantages over previous destructive methods which involve permeating compounds through a mucus filled device, and then taking sections in order to track compound permeation at a single timepoint.

Other approaches to mucus permeation include side-on three-compartment diffusion chambers, diffusion cells and Ussing chambers (162). A limitation of these systems can be mucus thickness, for example Bhat *et al.* used a 3,000 µm thick chamber which is considerably higher than *in vivo*, whereas Norris and Sinko used a mucus thickness of 380 µm. Furthermore Bhat *et al.* showed that the membrane material can cause significant variability in permeability data (168,169). This is a further advantage of using integrated systems that bring the mucus layer into direct contact with digestive secretions, and model epithelia.

While *ex vivo* studies of tissue samples can be limited by availability of material and are subject to donor variation, they naturally have advantages in terms of modelling the tissue architecture, and mucus thickness and composition (163). Intestinal loops and Ussing chambers are common methods for *ex vivo* tissue modelling.

Discussions and future directions

All 3D models described in this paper have proven to be useful in oesophageal research, with the 3D organoid presenting the most promising model, with a huge potential in personalised medicine. However, there is room for improvement, and this should be considered when further developing these models. While all models described may not contain all cells representative of a specific organ, and cell representation is somewhat variable between each culture, there is another major limiting factor - the lack of other, functional layers found deeper within the oesophageal tissue structure. This paper has already discussed the importance of the smooth muscle in the functions of the oesophagus, but there are important functions for immune cells, stromal cell types, neuronal control and a vascular system for nutrient transport and waste removal (92,114) which are not considered. Moreover, these deeper tissue layers can be subject to disease, such as ESCC or EADC tumour invasion (92). It is important to develop more complex oesophageal models in the future, which consider the latter points and contribute to a better mimicking of human oesophageal function, both in healthy and diseased oesophagi. There have been some successful developments in this area, with an example coming from Workman *et al.* [2017], who developed a human pluripotent-stem-cell-derived intestinal model to include a functional enteric nervous system by combining neural crest cells, which initiate mesenchymal differentiation into neuronal structures, and human intestinal organoids (129). Despite this model focusing on modelling the intestine, it shows promise for the utilisation of similar methods in the development of more complex oesophageal models.

An important area of future research will be to evaluate current and emerging models for use in modelling the action of reflux and the role this plays in disease pathology.

Through this paper we have identified limitations of modelling of the oesophageal and intestinal mucosa. Current research in our lab is based around development of fully integrated models that recapitulate the protective function and permeation characteristics of mucus to allow modelling of whole digestive secretions in cell culture systems that integrate a cell-compatible mucus barrier.

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