Chater PI, Wilcox MD, Pearson JP.

Efficacy and safety concerns over the use of mucus modulating agents for drug delivery using nanoscale systems.

***Advanced Drug Delivery Reviews* 2018, 124, 184-192**

Title Efficacy and safety concerns over the use of mucus modulating agents for drug delivery using nanoscale systems

Authors Peter I Chater[a], Matthew D Wilcox[a], Jeffrey P Pearson[a]

Affiliations [a]Institute of Cell & Molecular Biosciences, Newcastle University, The Medical School, Framlington Place, Newcastle upon Tyne. NE2 4HH, UK.

Corresponding Author Jeffrey P Pearson, Institute of Cell & Molecular Biosciences, Newcastle University, The Medical School, Framlington Place, Newcastle upon Tyne. NE2 4HH, UK. Jeffrey.pearson@ncl.ac.uk

Acknowledgements BBSRC Follow on Funding

Table of Contents

Table of Contents 2
1. Introduction 4
2. The delivery system? 8
2.1. Nanoparticles 8
2.1.1. A slippery surface 10
2.1.2. High density charged surface 10
2.1.3. Lipid derived delivery systems 12
2.1.4. Proteolytic enzyme coating 14
2.1.5. Reduction of disulphide bridges 15
2.1.6. Zeta potential changing methodology 16
2.2. Viral vectors, virus-like particles and nanocages 17
3. What about the mucus layer, how can it be modulated? 19
3.1. Mucolytic agents added prior to or with the delivery system 19
3.2. Agents that regulate mucus production and secretion 21
4. Mucus Models 22
4.1. Modelling GI delivery of Drug Delivery Systems 22
5. Safety concerns of mucus modulating delivery systems/agents 27
7. References 32

Abstract Drug delivery to the mucus covered mucosae is fraught with difficulties and many different approaches have been developed to permeate the mucus barrier. Generally by modifying the delivery system to avoid interaction with the mucus. These modifications are reviewed here in terms of efficacy and safety. These are particular problems for oral delivery the pharmaceutical industry's favoured route for drug administration. For effective delivery through the gastrointestinal tract a drug must pass through three barriers in sufficient amounts to yield a biological effect. These barriers are the digestive barrier in the lumen, the mucus barrier, and the epithelial barrier.

Other approaches involve mucolytic agents added with or prior to the delivery system or agents regulating mucus production and are reviewed here. In terms of safety, a key property of a mucus modulating delivery system is that it must not damage the protective function of the mucus layer.

Keywords Mucus; Toxicity; Efficacy; Drug Delivery; Modelling; Barrier Properties; Permeation

[1]Abbreviations

chitosan (C)
cowpea chlorotic mottle virus (CCMV)
cowpea mosaic virus (CPMV)
cystic fibrosis (CF)
long chain (LC)
medium chain (MC)
N-acetylcysteine (NAC)
no lipids (NL)
poly(lactic-co-glycolic acid) (PLGA)
periciliary layer (PCL)
6 phosphoglucuronic acid (PGA)
polyacrylic acid (PAA)
polyethylene glycol (PEG)
polyethylene imine (PEI)
porcine small intestinal mucus (PSIM)
pulsed-gradient spin-echo NMR (PGSE-NMR)
self-emulsifying drug delivery systems (SEDDS)
self-nanoemulsifying drug delivery systems (SNEDDS)
small angle neutron scattering (SANS)
spin-echo SANS (SESANS)

1. Introduction

The effective delivery of therapeutic agents to epithelial cells of mucus secreting mucosa and/ or the circulation beyond is hampered by the mucus layer which is designed to trap particles. These are then removed as the mucus layer is turned over. In terms of designing an effective system to overcome the mucus barrier we must first understand the 'enemy'. That involves understanding mucus structure and composition and its rate of turnover. If mucus turnover is faster than the drug carrier can penetrate the mucus layer then none will reach the epithelium. In terms of drug delivery the mucus covered epitheliums include the airways both upper and lower, the gastrointestinal tract excluding the mouth and the oesophagus which do not have an adherent mucus layer and are squamous rather than columnar epithelium [1]. Also considered as drug delivery targets are the mucus covered epithelium of cervico-vaginal tract and the ocular epithelium [2-5]. Mucus is 95% water and the gel forming constituent is the glycoprotein mucin [6]. IgA is actively secreted along with the mucin. The other components found in mucus result from shed cells e.g. DNA, actin and lipid. Particularly in the terminal ileum and the colon, microbial cell products contaminate the mucus. The makeup of mucus can be further altered in diseased states e.g. cystic fibrosis, with products from inflammatory cells leading to an increase of non-mucin components. Mucus gels once formed cannot be diluted out, but isolated gels will eventually be dissolved due to the action of endogenous degradative enzymes if these are not effectively inhibited [6]. Mucins consist of 3 groups; gel forming secreted, soluble secreted and membrane bound. Their structures are described in detail elsewhere [7, 8]. The gelling mucins consist of a highly glycosylated protein core with up to 90% of the weight being carbohydrate. These mucins form polymeric structures maintained by disulphide

bridges. The C and N terminals and bare or sparsely glycosylated regions of the protein core are open to proteolytic attack. Cleavage at these points will destroy the polymeric structure leading to solubilisation of the gel (Figure 1).

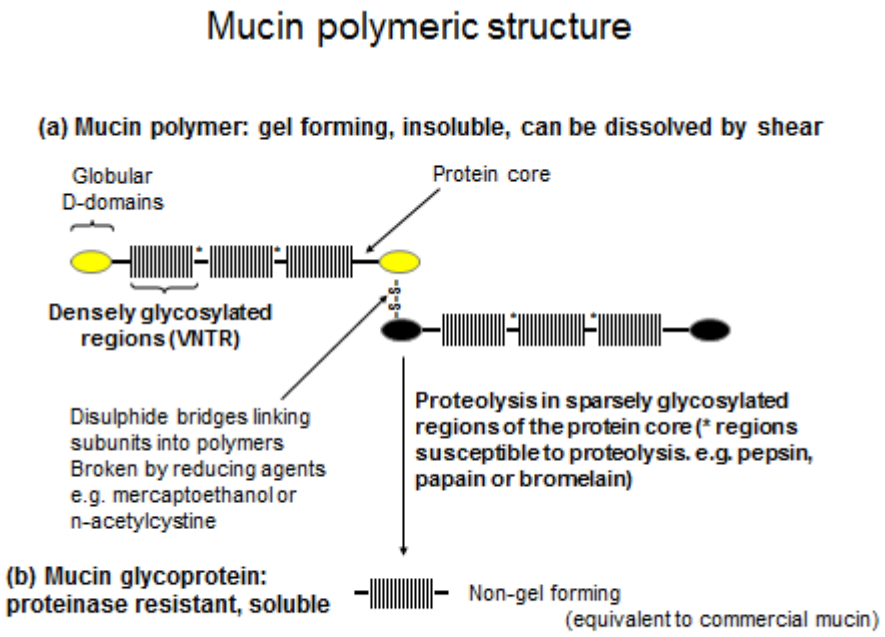

Figure 1 Mucin Polymeric Structure with variable number tandem repeats (VNTR)

The airways have a mucus barrier consisting of two layers; a layer close to the cells called the periciliary layer (PCL), in which the cilia beat and a mucus gel layer on top of the cilia. The PCL does not contain gel forming mucins but does contain membrane bound mucins expressed on the cilia surface and the epithelial cell apical surfaces. The identification of keratin sulphate (a glycosaminoglycan) in the PLC shows it is not simply a low viscosity fluid

[9, 10]. The reported pore sizes for these two layers are ~200nm for the mucus gel and between 6 and 40nm for the PCL [9]. Consequently if the drugs are to be delivered all the way to the cell surface then they would need to be in very small particles or in particles that release their cargo into the PCL after penetrating the gel layer.

Mucus in the GI tract is a bilayer, with a luminal layer which is shear compliant on the surface which overlies a shear resistant layer [11, 12]. There is some confusion as to clearance rates of these two mucus layers in the literature. It is common to read in reviews on mucus properties and drug delivery statements like; the non-adherent layer (which equates to the shear compliant layer) is rapidly transported or is rapidly cleared, whereas the underlying adherent (shear resistant) layer has a much slower clearance rate. Implying that drug delivery systems trapped in the upper layer will have short residency times. It is important to note that the shear compliant gel becomes a viscous liquid when put under low levels of shear but once the shear is removed it returns to a gel [11]. Therefore the drug delivery particles trapped in this layer would only be cleared quickly when shear is continuous i.e. in the fed state but not when shear is reduced in the fasting state. There is however a paucity of data on GI mucus clearance particularly considering the two layers.

In the lung rates are better characterised because it is possible to estimate clearance from cilia motion and has been reported as 5 mm/min with the mucus layer being removed every 20 minutes [5, 13]. This rate assumes no particles in the mucus and transport rates will depend on size of the particle and where it is in the lungs. Five mm/min will only be realistic

for the trachea but not the deep airways where the numbers of cilia are reduced [14]. Using a stochastically generated asymmetric model of the conducting airways Asgharian *et al.* [14] produced values for the deeper lung structure, with 1.0mm/min at 4 branches and 0.1mm/min at 8 branches. Therefore drug containing particles delivered to the deep lung will remain much longer, up to 24 hours in the peripheral bronchiolar airways [14].

The mucus layer of the eye is the thinnest of all the mucus covered epithelium, being between 0.2-1µm [15], consisting of a gel layer on top of membrane bound mucins. The turnover of the mucus layer is difficult to measure and values of seconds to hours have been quoted [3, 5]. Possibly the most accurate estimates have come from nanoparticle residency times on the eye with the assumption that they are binding to the mucus layer. Using this data mucus turnover times are around 40 minutes [2]. However some caution is needed is it is not always clear if the particles have penetrated into the cells.

Cervico-vaginal mucus is reported to be tens of µm thick and to have a turn over time of a few hours [5]. Unlike other mucus gels its physical properties are modulated during the menstrual cycle, when it becomes much less viscous during ovulation. This change would be expected to increase clearance rates of delivered drugs.

As the number of potential strategies and systems for delivery through mucus increases, the safety of these must be considered, in terms of the effect on the mucus layer and the underlying mucosa.

In this review we consider the safety and efficacy of the available mucus modulating drug delivery systems and consider the gaps in our current knowledge.

2. The delivery system?

2.1. Nanoparticles

Nanoparticles can be defined as particles with at least one dimension 100nm or less and having a high surface to volume ratio [16]. The first consideration is size. Nanoparticles have the potential to penetrate the mucus layer if they are below the range of the mucus pores ~ 200nm [17]. However surface characteristics may mean they interact with the mucus and do not pass through. Several modifications have been applied to nanoparticles to enhance their mucus penetrating properties (Table 1).

Table 1 Approaches to permeating the mucus layer

Approaches to permeating the mucus layer	Effective agent	Mucus Layer	Ref
Slippery Surface	PEG, Methyl silicones	Native Healthy	[18, 19]
High density charged surface with approx. same number of +ve and –ve charges to give a net charge close to neutral	Polyacrylic acid/polyallylamine Arginine-glutamic acid	Native Healthy	[20, 21]
Neutral to low surface charge	Poly acrylic acid, chitosan, dextrans, cyclodextrans	Native Healthy	[20]
SNEDDS / Liposomes	Hydrophobic surface and small size mixture of glycerides, triglycerides and surfactants	Native Healthy	[22-25]
Disulphide bond breakers released from the nanoparticle or used in conjunction with the delivery system	N-acetylcysteine, mercaptobenzoic acids, Dithiotheitol	Native Healthy	[26]
Proteolytic (mucolytic) enzymes attached to the NP surface	Papain, trypsin, Bromelain	Native Healthy	[27, 28]
Thiomers that change reactivity depending on pH	Polyacrylic acid, cysteine conjugates	Native Healthy	[29]
Nanoparticles with surface charge changing characteristics	Polyacrylic acid, n-acetyl amino acid, hydrophobic amino acids side chains	Native Healthy	[25, 30, 31]
Surface decorated nanoparticles with DNase to increase permeability	DNase	Disease state CF mucus	[32, 33]
Altering mucus layer permeability	Gelsolin	Disease state CF mucus	[32]
Agents that regulate mucus production and secretion	Glycopyrrolate, prostaglandin synthesis inhibitors	Healthy and Diseased	[34-36]
Mucoadhesive particles (1) in conjunction with Mucopermeating particles (2)	(1) Amine modified polystyrene nanoparticles, (2) PEG coated polystyrene particles	Healthy (prolonged storage at 4^{o}C)	[37]
Particles engineered with both mucoadhesive and mucopermeating properties	PEG/Chitosan	Mucus suspension	[38]
Proteolytic enzymes plus a disulphide bond breaking agent	Papain and cysteine	Native healthy	[27]

2.1.1. A slippery surface

One way this can be achieved is to coat nanoparticles with polyethylene glycol (PEG). It has been demonstrated that non-penetrating, mucoadhesive, particles can be converted to mucopenetrating by coating them with short chain PEG between 2,000-5,000 molecular weight. Carboxyl modified polystyrene nanoparticles with a surface charge of -33 to -69mV did not permeate the mucus layer. However when coated with short chain PEG the charge was increased to between -2.2 to -4.0mV they did permeate. When the PEG modified nanoparticles were applied to the mucus surface of mice vaginal explants they reached the mucosal surface within 10 minutes [18, 19]. It appears that PEG size is critical for this enhanced mucus permeating effect, as particles coated with 10,000 molecular weight PEG were trapped in the mucus. It has been suggested that the longer chains of PEG can penetrate into the hydration sphere of the mucins and form hydrogen bonds [39, 40].

2.1.2. High density charged surface

Based on the fact that virus particles, e.g. the Norwalk virus, can rapidly permeate human cervical mucus gels [4, 41], nanoparticles have been designed to mimic the virus surface characteristics. The Norwalk virus is small, 38nm and has a high surface density of negative and positive groups, made up of acidic and basic amino acids. So the surface charge will depend on the pH environment. The isoelectric point for this virus is around 4 so it will be around neutral at pH between 3-5 [42, 43]. Consequently because cervical mucus has an

acidic pH [4] the virus will be near neutral and can pass rapidly through the mucus layer. This however may not be the case with a mucus layer with a pH near to 7 where the virus would have a negative surface charge. Polyacrylic acid and polyallylamine nanoparticles have been produced with an overall surface charge of +0.9mV. These nanoparticles diffused around 2x faster than nanoparticles made with polyacrylic acid alone or polyallylamine alone in native porcine small intestinal mucus [21]. Abdulkarim *et al.* [20] produced a series of polyacrylic acid (PAA)/chitosan (C) nanoparticles with zeta potentials ranging from -29 to +19.5 mV depending on the ratios of PAA:C. At a ratio of 1:2.2 a near neutral particle with a zeta potential of +1.1 ±2.4mV was produced. Its diffusion was measured in porcine small intestinal mucus using multiple particle tracking and compared to negative, positive nanoparticles, adenovirus AD5 and a PEG-PLGA nanoparticle. In order to account for the different sizes of particles their diffusion rates were determined by dividing the diffusion coefficient in mucus by the coefficient in water. Based on this ratio the near neutral was 1.5X faster than the AD5 viral particle (-0.5 ±2.3mV) but the PEG-PLGA (-8.3 ±1.2mV) was still 2.6X faster than the near neutral particle. However when compared to the particle made from 1:8, PAA:C (+19.2 ±0.6mV) the near neutral particle was 89X faster and 18X faster than the particle made from 4:1, PAA:C (-30.6 ±4.4mV). When compared to PLGA alone the PEG-PLGA was more than 1000X faster and the near neutral particle was 430X faster. The ability of a near neutral particle to permeate mucus effectively relates to lack of any charge: charge interaction with the mucins and because these particles have high levels of charge there will be reduced hydrophobic interactions with the mucus. These diffusion results indicate the effectiveness of near neutral particles in permeating mucus layers and their potential as drug delivery agents. However, one problem with the near neutral

particles is their tendency to form aggregates which means they require sonication before use.

2.1.3. Lipid derived delivery systems

Cationic lipid based liposomes have been produced as their positive charge is useful in interacting with a negative DNA cargo in gene delivery and the hydrophobic lipid part can interact with the cell membrane enhancing uptake via caveolae and clathrin mediated pathways. Efficient transfection can take place if no intact mucus layer is present [23, 44, 45]. Positively charged liposomes will be strongly adhesive to the mucus layer [20, 44, 45]. Neutral liposomes made from cholesterol and phosphatidylcholine again are trapped in the mucus layer due to hydrophobic interactions. This can be overcome by coating with PEG 2000, giving a nanoparticle of 181mn a zeta potential of -13mV, which when loaded with interferon alpha gave enhanced delivery of the cargo to an *ex vivo* sheep vaginal epithelium [23]. The size of the liposomes was shown to be important in terms of cellular uptake. A study by Li *et al.* [46] showed that negative liposomes made from phosphatidylcholine and medium chain triglycerides (zeta potential ~-50mV) were taken up effectively by Caco-2 cells. With nanoparticles of 100nm being better than 200nm which were both better than 300nm. However in the *in vivo* studies it was not clear if the nanoparticles had passed through the mucus layer or been trapped in it. One might expect particles with such a large negative charge would be repelled by the mucus layer rather than pass through it. In addition both these papers [23, 46] determined nanoparticle interaction with the mucus barrier using mucin interaction models with a degraded mucin not representative of the *in vivo* mucus gel [17, 47].

A more promising approach using lipids has come with the development of self-nanoemulsifying drug delivery systems (SNEDDS) which are emulsions of oils and surfactants, formed on mixing with water, producing small sizes nanostructures. The sizes range from 12-455nm [22, 25]. In the study by Zupancic *et al.* [25] the size of the SNEDDS depended on the lipid component. Long chain lipids (> C_{10}) and medium chain lipids (C_8-C_{10}) produced droplets of 30-240nm and short chain lipids (< C_8) produced droplets of between 165-265nm. When lipid was excluded from the formulation droplets of 13-68nm were produced.

SNEDDS have hydrophobic surface properties which could lead to interaction with hydrophobic regions in mucus. As particle size is an important parameter in governing mucus permeation [48] and to avoid hydrophobic interactions SNEDDS of small size which have space to spare in the mucus pores will permeate the fastest. In addition SNEDDS have the ability to deform and change shape to fit through convoluted pores. Consequently SNEDDS with short chain lipids were excluded from the mucus diffusion studies. When SNEDDS containing long chain (LC) lipids, medium chain (MC) lipids and no lipids (NL), all in the size range 30-40nm were compared in their ability to permeate a mucus gel, no lipid and medium chain lipid were 2x faster than long chain (LC) lipid SNEDDS. This can be explained by the LC SNEDDS having a greater capacity for hydrophobic interactions. This enhanced permeation was also exhibited *in vivo* by MC and NL SNEDDS containing the anticoagulant enoxaparin. Experiments in rats showed that enoxaparin could be delivered to the circulation orally when contained in MC and NL SNEDDS with an absolute bioavailability of

~2% compared to zero for an orally delivered aqueous enoxaparin solution [25]. Another positive for the use of SNEDDS as an oral delivery system is their ability to protect the cargo from degradative digestive enzymes. SNEDDS (30-40nm) have been developed to incorporate insulin as an insulin/dimyristoyl phosphatidylglycerol hydrophobic ion pair and have been shown to protect the insulin from proteolytic digestion by the pancreatic enzymes trypsin and chymotrypsin [24].

2.1.4. Proteolytic enzyme coating

Another approach to permeating the mucus barrier is by immobilizing on the surface of particles, proteolytic (therefore mucolytic [28]) enzymes e.g. papain. The enzyme is attached via covalent interactions between amino groups on the enzyme and carboxyl groups on polyacrylic acid using carbodiimide chemistry. Using this technology Muller *et al.* [27] showed that in a transwell system, incorporating porcine small intestinal mucus, that papain decorated nanoparticles permeated 3 times faster than the naked polyacrylic acid nanoparticles. In addition, if cysteine was added to the particle, papain being a thiol dependent enzyme, then the nanoparticles improved their permeation to 3.5 times faster than the naked nanoparticle. Demonstrating that these decorated nanoparticles would effectively enhance drug delivery. The authors further showed, in an oral delivery rat study, that papain nanoparticles had an increased residency time in the jejunum after 3 hours, presumably because they had reached the mucosa under the mucus layer [27]. Nanoparticles have been manufactured with other proteolytic enzymes e.g. bromelain and trypsin linked to PLGA and these again showed enhanced permeation of porcine small intestine mucus, with bromelain being better than trypsin [49]. Most likely due to the

enhanced mucolytic activity of bromelain compared to trypsin. In a further study [50] the movement of proteolytic enzyme decorated nanoparticles was studied using a rotating tube containing porcine small intestinal mucus [51] and the effect on the mucus layer measured using pulsed-gradient spin-echo NMR (PGSE-NMR), small angle neutron scattering (SANS) and spin-echo SANS (SESANS). Bromelain decorated nanoparticles showed the best permeation into the mucus layer with 4.8 times more reaching 6mm into the mucus than papain decorated nanoparticles. This was to some extent explained by the fact that bromelain survived the production of the enzyme polymer conjugate better and retained 76% of its enzyme activity compared to 43% for papain. Using a porcine small intestinal mucin gel PGSE-NMR demonstrated an increase in mucin diffusion caused by the enzyme linked nanoparticles, with bromelain having the biggest effect. These changes were confirmed in the porcine small intestinal mucus gel by SANS and SESANS and suggests the breakdown of polymeric structure (Figure 1). It is important to note that although bromelain linked nanoparticles are better at penetrating mucus the bigger changes in mucus structure could compromise its mucosal protection. Consequently these enzyme linked nanoparticles should be designed to pass through the mucus layer without too much perturbation of its structure.

2.1.5. Reduction of disulphide bridges

As the gel structure of mucus depends on mucin polymeric structure, agents which reduce disulphide bridges such as mercaptoethanol or dithiothreitol have been widely used in research and the clinic [52, 53] to dissolve mucus gels by destroying the disulphide based mucin polymeric structure. Little research has been carried out to attach –SH containing

groups to the outside of drug delivery systems in order to cleave the mucin disulphide bridges and completely penetrate the mucus layer. Most research has concentrated on a thiomer strategy [29] using the pH gradient in the small intestine, an acid microclimate, pH 6.0 in the lumen to pH 7.4 close to the epithelial cell surface, which is the same microclimate that powers absorption of peptides. The reactive species generated from thiol groups is S^- (thiolate anion) and it is this that can drive disulphide exchange forming disulphide bridges between the nanoparticle and the mucin molecules. The amount of this anion depends on the pKa of the thiol ligand e.g. N- acetylcysteine and cysteine have pKa's of 8.2 and 8.4 respectively, meaning that a change in pH from 6 to 7.4 would cause a significant increase in disulphide exchange. This would mean that nanoparticles with n-acetylcysteine of cysteine attached to the surface would not react with the mucin at the luminal mucus surface but penetrate close to the cell surface where they would become immobilized by interaction with the mucin [54]. Where thiols could increase permeation without mucin binding is when nanoparticles have cysteine and papain attached to the surface. This combination has been shown to increase their permeation of mucus. This was explained by cysteine enhancing the mucolytic activity of papain [27]. However an alternative explanation is that cleavage of mucin disulphide bridges could be the reason for the enhanced permeation.

2.1.6. Zeta potential changing methodology

Based on the data showing negatively charged nanoparticles permeated mucus better than positively charged ones and positively charged nanoparticles are taken up by cells better than negatively charged ones [22, 55] a zeta changing strategy has been developed. This

methodology can be based on the cleavage of a peptide bond by membrane endopeptidases. The initial nanoparticles have a negative zeta potential allowing permeation of the mucus layer and have conjugated to their surface a peptide containing hydrophobic amino acids. When the nanoparticles reach the cell surface the peptide is cleaved between hydrophobic amino acids leaving a positively charged amino group exposed resulting in a change in zeta potential to positive. Another approach is to link glutamic acid to the surface giving a net negative charge and this can be cleaved of at the cell surface by γ-glutamyl carbopeptidase resulting in a net positive charge [25]. A third approach was developed by Suchaoin *et al.* [31] and Bonengel *et al.* [30] where phosphate containing polymers were incorporated into nanoparticles to give rise to a negative zeta potential. These were converted to a positive zeta potential by the action of intestinal alkaline phosphatase. The nanoparticles were self-emulsifying drug delivery systems (SEDDS) one with 1, 2 dipalmitoyl-sn-glycero-3-phosphatidic acid containing a terminal phosphate group and another with carboxymethyl cellulose/polyethylene imine (PEI) nanoparticles with 6 phosphoglucuronic acid (PGA) attached containing a terminal phosphate group. Both these nanoparticles could be converted from negative to positive by the action of alkaline phosphatase when they were exposed to Caco-2 cells in culture. This *in vitro* culture work suggests that this type of nanoparticle could be effective *in vivo* but as yet there is little *in vivo* data to confirm this.

2.2. Viral vectors, virus-like particles and nanocages

These vectors have greater efficacy in terms of gene delivery than conventional nanoparticles. Many studies have been carried out in the lung involving gene therapy (see Di

Gioia *et al.* for review [44]). Viral particles can penetrate the mucus layer because of their small size e.g. cowpea mosaic virus (CPMV) (31nm), cowpea chlorotic mottle virus (CCMV) (18nm) and bacteriophage MS2 (27nm) [56] and near neutral surface charge. They do not cause changes to the mucus layer as they pass through it but when they reach the target cells can induce strong immune responses including cytokine release from the epithelial cells. Toxicology studies in mice demonstrated that CCMV produced a large IgM and IgG response and CPMV showed a general increase in the immune response [57, 58]. Cytokine release will stimulate mucus secretion increasing the mucus barrier thickness; slowing the rate of cargo delivery from subsequent dosing [59]. In general nanoparticles are safer than viral vectors. Nanocages based on ferritin (an iron transport protein which would not promote an immune response in humans) can be formed with a central space which can be loaded with an API [60]. It can then be surface coated with PEG to allow penetration through the mucus layer *in vivo*. The added value of the ferritin presence in the nanoparticle is that its receptor is highly expressed in many tumour cells [61]. So Any API should be selectively taken up by cancer cells. Huang *et al.* [62] loaded the cages with doxorubicin and delivered these to the lung by intranasal instillation. The doxorubicin loaded nanocages improved survival compared to doxorubicin solution alone in a mouse lung cancer model.

2.3. Combinations of drug delivery strategies

Wang *et al.* [37] investigated a two particle strategy, a mucoadhesive particle to interact with the mucins in the mucus gel leading to an enlargement of the pores in the gel. The formulation could then contain a mucopermeating particle which would pass unhindered through these enlarged pores. Another two strategy approach was adopted by Sharma *et al* [38] using PEG conjugated chitosan nanoparticles loaded with soluble telmisartan, an

angiotensin II receptor blocker. This gave a balance between mucoadhesion by chitosan and the mucopenetrative properties of PEG, in the hope of retaining the nanoparticles in the deeper layers of the mucus and closer to the epithelium.

Another potential dual strategy is where nanoparticles decorated with papain can permeate the mucus layer faster when cysteine is present. This was explained by the authors as cysteine activation of the enzyme [27]. An alternative explanation is that permeation was enhanced due to the disruption of mucin-mucin disulphide bridges by the action of the thiol containing amino acid.

3. What about the mucus layer, how can it be modulated?

3.1. Mucolytic agents added prior to or with the delivery system

Mucolytic agents such as N-acetylcysteine (NAC) have been used to enhance drug delivery in the intestine. Takatsuka *et al.* [26] demonstrated that treatment of exteriorised rat jejunum with 5% NAC and 5% non-ionic surfactant enhanced the uptake of calcitonin. However, this lead to acute mucosal damage as demonstrated by necrotic villi and shortening of villus height. This damage was reversible within 2 hours post administration. However, even short time exposure of the mucosa could allow pathogen access [63]. This approach has problems as the rats were fasted and the jejunum exteriorised, incised, and solutions were administered through an adhered delivery device. Consequently the mucosa was not exposed to the normal range of gastric and pancreatic digestive fluids which could lead to much more damage. It is therefore very important to consider digestive fluids in any damage model.

Broughton-Head *et al.* [32] have considered the use of "mucolytic agents" in enhancing drug delivery through a diseased mucus layer. In cystic fibrosis (CF) the airway mucus layer is dehydrated and thickened due to inflammatory responses to pathogenic infections and contains larger quantities of DNA and actin [64, 65]. Consequently agents that act on all the biopolymers present including mucin could enhance drug delivery either to the mucosa or to bacterial colonies within the mucus. The study [32] used combinations of these biopolymers to generate CF mucus like material and measured the diffusion of 200nm fluorescent carboxylated nanospheres. As expected mixtures of mucin and DNA retarded the diffusion of the nanospheres compared to a PBS control and the diffusion rates returned to control levels with DNase or NAC treatment. Addition of F-actin did not retard the nanospheres any more than DNA and mucin mixtures. It did however inhibit the ability of DNase or NAC to enhance the diffusion. Depolymerising the actin with gelsolin did not correct this inhibition. This implies that the presence of actin in any state of polymerisation can inhibit the ability of conventional agents used as mucolytics in CF to enhance penetration into the mucus layer. Some caution must be applied when extrapolating this data to the CF lung as the mucin used was a commercial preparation which does not have the gel forming properties of native mucus [17]. This may explain why the mixture of actin, mucin and DNA at the concentrations found in CF airway mucus did not retard diffusion of nanospheres as much as CF sputum, 53% and 96% respectively [32]. The need to deliver antibiotics effectively in cystic fibrosis has led to attempts to improve antibiotic delivery to a key bacterium, *Pseudomonas aeruginosa* [66]. To achieve this they linked DNase to the outside of nanoparticles containing tobramycin an antibiotic effective against *Pseudomonas* [33]. The authors generated a model of nanoparticle penetration by overlaying a gelatin layer with cystic fibrosis sputum and applying fluorescently labelled nanoparticles to the

sputum surface. After 24 hours the fluorescence levels in the gelatin were measured and equated to the percentage of nanoparticles had permeated the sputum. Tobramycin has serious dose related side effects, e.g. nephrotoxicity and ototoxicity [67] and a narrow therapeutic index.

Consequently a great treatment advance could be achieved if it could be packaged in nanoparticles that could penetrate the mucus to the site of the infection and release the antibiotic, generating high local concentrations with antimicrobial effects and greatly reduced systemic effects. The study showed that alginate-tobramycin complexes could be successfully formed into nanoparticles with chitosan and surface coated with DNase linked via a carbodiimide reaction and could successfully penetrate cystic fibrosis mucus and kill *Pseudomonas* colonies within the mucus.

3.2. Agents that regulate mucus production and secretion

Drugs such as glycopyrrolate block parasympathetic nerve activity which normally stimulates mucus secretion in the airways. Glycopyrrolate and similar drugs may be useful when the mucus layer is thickened by disease related hypersecretion [68] but may expose the mucosa to potential insult in the normal situation. It is well documented that prostaglandins stimulate the production of normal mucus levels in the gastrointestinal tract [35, 36]. Inhibition of prostaglandin synthesis by compounds such as non-steroidal anti-inflammatory drugs (e.g. indomethacin) and by glucocorticoids [34] could be used to compromise the mucus layer to allow effective drug access to the mucosa. However this

could only be attempted in the acute situation as long term prostaglandin inhibition leads to gastric damage.

4. Mucus Models

4.1. Modelling GI delivery of Drug Delivery Systems

Robust *in vitro* models can provide useful tools for assessing safety and efficacy which can complement, reduce and occasionally replace *in vivo* models, however aiming to accurately simulate the passage of delivery formulations through the gastrointestinal tract *in vitro* carries a number of challenges.

In oral delivery of particle formulations, there are three 'barriers' which must be considered; the digestive barrier, the mucus barrier and the epithelial barrier.

Methods exist to model the digestive, mucus permeation and epithelial phases in isolation however there are a number of challenges to integrate modelling of these phases into a single system. Small intestinal fluids contain proteases and components of bile which are toxic to cells. The normal function of mucus *in vivo* protects the underlying epithelia from damage by these digestive fluids, however replicating this effect *in vitro* has proved challenging.

Due to ease of use, cost, reproducibility and positive correlations with *in vivo* data, Caco-2 cell lines have become the most widely accepted model for *in vitro* modelling of small intestinal permeability [69]. Caco-2 cells are highly differentiated with appropriate

morphology, brush border enzyme expression, tight junctions and can be used to model active and passive transport as well as efflux [69]. However Caco-2 monocultures produce no significant mucus layer and so cannot be combined with simulated digestive fluids. Appropriate composition of digestive fluids has been discussed in Minekus *et al.* [70].

Furthermore, the mucus barrier *in vivo* may keep delivery systems from direct contact with the epithelia, while allowing them in close enough proximity to deliver their payload. In direct application of the delivery systems to a cell-culture system, this protective barrier effect would not be replicated, and cytotoxic effects of delivery systems such as those decorated with proteases may be overestimated.

Incorporation of a mucus barrier is of 'key importance' to evaluating mucosal drug permeation [71]. Caco-2/HT29-MTX co-cultures have been adopted to overcome the lack of a mucus layer. However the mucus layer produced with Caco-2/HT29MTX co-culture is 2-10µm thick, as compared to an *in vivo* thickness of up to 400-500µm [72]. This means that even co-culture systems cannot be integrated with whole digestive fluids due to cell death.

Some gastrointestinal models, such as the TIM model designed by Minekus *et al.*,[73] aim to mimic the absorptive phase through dialysis across a semi-permeable membrane to simulate small intestinal uptake. The authors argue that this 'closely approximates absorption of nutrients through the lumen of the gut'; although it is understood elsewhere that intestinal absorption is somewhat more complex than size exclusion diffusion.

Methods have been developed to test the permeation of nanoparticle delivery systems such as SNEDDS through an intestinal mucus layer in a transwell set-up [74]. However, while

providing a useful model of mucus permeation, the non-sterile native mucus used in these systems would not be compatible with a cell culture system [71].

Attempts have been made to use 'biosimilar' mucus which can be combined with cell culture systems without causing cell death. Boegh *et al.* [71] showed that porcine intestinal mucus disrupted a Caco-2 monolayer, and therefore adopted a 'biosimilar' mucus composed of degraded gastric commercial mucin, bovine serum albumin, cholesterol, phosphatidylcholine, linoleic acid and the non-natural component polyacrylic acid, which was responsible for steric barrier properties in the model. However, in their own research, significant differences in peptide permeability were shown between porcine intestinal mucus and biosimilar mucus.

Methods such as the M-SHIME large intestinal model from ProDigest have adopted a compartmentalised approach to work around the problems of integrating a mucus layer with a cell culture system. In this model, an enterocyte monolayer is grown in the lower compartment, and in the upper compartment, an artificial mucus layer is applied to a semi-permeable membrane. This means bacteria can be grown in the 'luminal' chamber, and the products of both the epithelial and bacterial cultures can freely diffuse across the semi-permeable membrane without the artificial mucus or luminal contents coming into direct contact with the enterocyte culture. Furthermore the artificial mucus used in this system is a mucin-agar mix (Figure 2).

We were only able to find limited information about the methodology, however, in Abeele *et al.* [75] and Marzorati *et al.* [76], Sigma-Aldrich porcine gastric mucin type II was mixed 5% (w/v) with agar and sterilised by autoclaving at 121°C. The limitations of Sigma-Aldrich mucin, and other commercially available mucins have been discussed elsewhere, as the

action of proteases during the isolation process damages mucin structure and effects mucin-mucin interaction, destroying their ability to form gels [77]. We were unable to find literature regarding the effects of autoclaving on mucin; we are tempted to speculate that no one has felt these experiments were necessary to show the effects on mucin structure would be catastrophic.

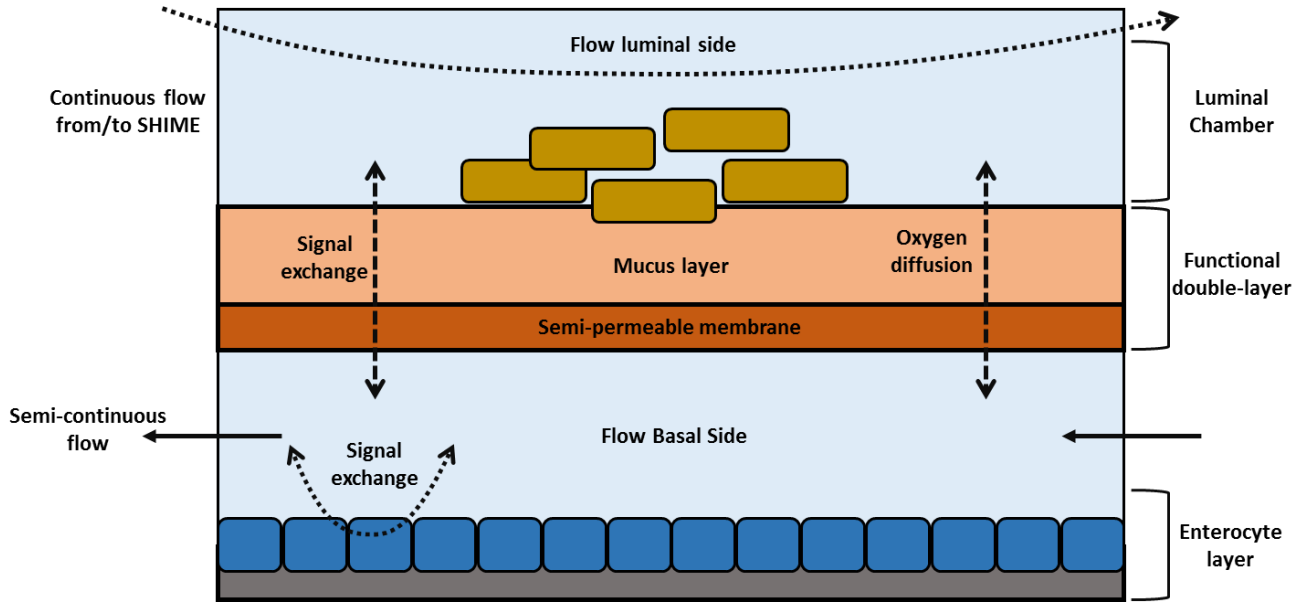

Figure 2 Adapted from Marzorati *et al.* [78]. Schematic diagram of an 'adhesion unit' for the study of study of microbial growth and host-microbiota interaction. The functional 'mucus' layer consists of a layer of agar-mucin mix which has undergone autoclaving, which is separated from an epithelial culture in the basal chamber by a semi-permeable membrane. The luminal chamber is supplied with continuous flow to/from the SHIME model.

Unpublished work in our lab has made significant progress in combining a purified and rebuilt mucus layer directly with cell-culture systems without compromising cell viability (at 5 hours). Furthermore this mucus layer contains no artificial components and can afford

protection from whole digestive fluids complete with bile, proteases, carbohydratases and lipases.

A number of model gut systems have been developed to study different aspects of digestion and GI physiology. These include; bioavailability and bioaccessibility of contaminants [79], digestion of allergens [80] study of pre and probiotics [81], models of gut motility, peristaltic motion and physiological mixing and shearing [82], enzymatic digestion[82], substrate digestion and interaction [83], intestinal microbiota [84], water and nutrient absorption [85] and drug delivery [86].

Wickham *et al.* 2009, define the three stages of digestion which upper GI models must consider; "(i) processing in the mouth, (ii) processing in the stomach (cumulative to the mouth) and (iii) processing in the duodenum (cumulative of the mouth and stomach)" [87]. Blanquet *et al.* 2004, define the importance of accurate modelling of the physiological composition of the digestive fluids, and accurate simulation of the physical forces and uptake processes which digesta is subject to; "(i) sequential use of enzymes in physiological amounts, (ii) appropriate pH for the enzymes and addition of relevant cofactors such as bile salts and coenzymes, (iii) removal of the products of digestion, (iv) appropriate mixing at each stage of digestion, and (v) physiological transit times for each step of digestion." [88].

To add to these definitions we would argue that a robust *in vitro* model for testing oral drug delivery of nanoparticles, as well as meeting the above criteria for the digestive phase should also include a non-artificial mucus layer which retains the rheological properties of native mucus, applied directly to a representative cell culture system.

5. Safety concerns of mucus modulating delivery systems/agents

In order to effectively penetrate the mucus barrier nanoparticles have been surface coated to give near neutral/negative zeta potentials and as such are less cytotoxic to non-phagocytic cells such as absorptive mucosa but are more cytotoxic to phagocytic cells than positively charged nanoparticles [89].

PEG coating can make positive particles neutral on the surface and thereby enhance mucus permeation. PEG coating of positively charged nanoparticles also reduces cytotoxicity as shown with human cervix epithelial carcinoma cells [90]. Extensive safety testing in animals has demonstrated LD50 values of 2g/Kg for PEG delivered via an oral route and no evidence of genotoxicity or carcinogenicity [91].

Poly anhydride nanoparticles have been developed for drug delivery in several bacterial diseases and in targeted delivery in cancer [92-94] but are adaptable for oral delivery in many diseases. They can be designed for oral delivery with reduced toxicity of the payload drug and can be surface modified to enhance mucus permeation. They have the further advantage of being biodegradable with low levels of toxicity [95]. Iglesias *et al.* [96] demonstrated that poly anhydride nanoparticle surface modified with PEG , mannosamine, 2-hydroxypropyl –β-cyclodextran, dextran or aminodextran producing a negative zeta potential demonstrated enhanced mucus permeation. In addition they had low toxicity with concentrations up to 10mg/ml with Caco-2 cells, detected using ATP and membrane integrity assays. Concentrations of 2 mg/ml showed no effect on cell proliferation or DNA

damage (genotoxicity) using an alkaline comet assay in combination with the enzyme formamidopyrimidine DNA-glycosylase [97].

PLGA nanoparticles are biodegradable and show low cytotoxicity even up to 300mg/ml [98] with a macrophage and an epithelial cell line. However at 300mg/ml there was a 3 fold increase in the levels of the inflammatory cytokine inflammation TNFα. This was size dependent with small particles being more inflammatory due to the binding protein. PLGA nanoparticles can be made more negative by the addition of poloxamer for example but this can also increase cytotoxicity as shown with Calu-3 lung epithelial cells. This effect appears to be due to the poloxamer directly [99].

As all absorptive mucosa contain immune cells such as phagocytic macrophages which are important in preventing microbial invasion. Penetrating to the mucosa with negatively charged nanoparticles could compromise mucosal protection as they are cytotoxic to macrophages.

A two particle strategy was proposed by Wang *et al.* [37] with high concentrations of particles that strongly adhered to mucus and opened out the pores in the mucus layer, increasing the permeation of a PEG coated nanoparticle. The potential danger of this approach is that it may increase microbial access to the mucosa through these enlarged pores.

Two delivery systems have been combined by Hetenyi *et al.* [29] (SEDDS and thiomers), in this case a thiolated amphiphilic polymer. This combination was seen to improve permeation and increased residency time on/or close to the mucosa. However based on cytotoxicity studies with Caco-2 cells this delivery system showed significant cell death at high concentrations i.e. a 1:100 dilution. Although the concentrations in the lumen of the digestive system from oral delivery would be below this level it is not known if these toxic concentrations could arise locally at the mucosal surface *in vivo*.

Another mucus permeating method is zeta potential changing systems. However, the cytotoxicity of the surface entities that are modified by enzymatic action need to be carefully assed. For example PEI-6PGA covered carboxymethyl cellulose particles at 1mg/ml caused ~70% cell death of Caco-2 cells and the surface components PEI almost 100% and 6PGA~70% at equivalent concentrations [30].

SEDDS have also been modified with 1, 2 dipalmitoyl-sn-glycero-3-phosphatidic acid to generate a zeta potential changing system. Again high concentrations of these SEDDS at a dilution of 1:100 led to an 80% loss of Caco-2 cell viability [31].

In addition the composition of the SEDDS can make them more or less toxic. Karamanidou *et al.* [24] showed that using lauroglycol FCC in their SEDDS led to a reduction in safe concentration from 2mg/ml to 0.25mg/ml.

A note of caution should be applied to cytotoxicity measurements when selecting drug delivery systems as safe to proceed to animal studies and clinical trials. The most commonly used cytotoxicity test involves MTT (tetrazolium dye assay) [100] and modifications of this assay which measure mitochondrial function via the activity of mitochondrial enzymes which produces a coloured product. The cells used are transformed cell lines which are more resistant to toxic agents than primary cells which more closely resemble *in vivo*. It has been shown that there are several ways the delivery system can interfere with the assay: 1. giving a background absorbance; 2. binding to the coloured product; 3. having surface oxidative properties [101].

A key property of a mucus modulating drug delivery system is that the mucus layer retains its protective function after penetration. Wilcox *et al.* [102] demonstrated that nanoparticles decorated with proteolytic enzymes could penetrate small intestine mucus without altering its global rheological properties. The properties of mucus being able to flow and reanneal when ruptured [103] would mean that the pores produced by the proteolytic enzymes would seal relatively quickly. This would not be the case with total removal of the layer with free proteolytic enzymes or reducing agents like NAC, where the mucosa would remain unprotected until a new layer is secreted. Another potentially detrimental situation

is the opening up of pores with mucoadhesive particles [37] as this will prevent the ability of the mucus to flow and refill the enlarged pores.

6. Conclusions and future perspectives

There are many new developments in drug delivery systems targeted to overcome the mucus barrier which covers most potential drug delivery sites and most have shown enhanced permeation of mucus. The problem is that most of the new formulations will require extensive safety and clinical trials before they could be used in the clinic. In general there is a lack of toxicological data in primary epithelial cell and this must be addressed.

A further large gap in our understanding is the turnover rates for mucus *in vivo* which can potentially vary at different sites within the same organ. Also mucus turnover in the GI tract will vary on the fed vs fasting situation. The presence of food will increase the shear on the surface of the mucus layer, some of which will mix with the food bolus to lubricate its passage through the gut. In addition there is little or no data on the differential turnover of the shear compliant and the shear non-compliant mucus layers. This needs to be determined so that accurate modelling can be made of drug delivery system transit rates.

Finally it is imperative to develop physiologically relevant *in vitro* systems that reliably model the supra-mucus barriers, mucus and cell barriers to delivery.

7. References

[1] J. Dixon, V. Strugala, S.M. Griffin, M.R. Welfare, P.W. Dettmar, A. Allen, J.P. Pearson, Esophageal mucin: An adherent mucus gel barrier is absent in the normal esophagus but present in columnar-lined Barrett's esophagus, American Journal of Gastroenterology, 96 (2001) 2575-2583.
[2] A. Zimmer, J. Kreuter, Microspheres and nanoparticles used in ocular delivery systems, Advanced Drug Delivery Reviews, 16 (1995) 61-73.
[3] L.R. Schopf, A.M. Popov, E.M. Enlow, J.L. Bourassa, W.Z. Ong, P. Nowak, H.M. Chen, Topical Ocular Drug Delivery to the Back of the Eye by Mucus-Penetrating Particles, Translational Vision Science & Technology, 4 (2015).
[4] L.M. Ensign, R. Cone, J. Hanes, Nanoparticle-based drug delivery to the vagina: A review, Journal of Controlled Release, 190 (2014) 500-514.
[5] S.K. Lai, Y.Y. Wang, J. Hanes, Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues, Advanced Drug Delivery Reviews, 61 (2009) 158-171.
[6] A. Allen, Structure and function of gastrointestinal mucus in: J.L. R (Ed.) Physiology of the gastrointestinal tract, Raven press, New York, 1981, pp. 617-640.
[7] A.P. Corfield, Mucins: A biologically relevant glycan barrier in mucosal protection, Biochimica Et Biophysica Acta-General Subjects, 1850 (2015) 236-252.
[8] J. Dekker, J.W.A. Rossen, H.A. Buller, A.W.C. Einerhand, The MUC family: an obituary, Trends in Biochemical Sciences, 27 (2002) 126-131.
[9] M. Kesimer, C. Ehre, K.A. Burns, C.W. Davis, J.K. Sheehan, R.J. Pickles, Molecular organization of the mucins and glycocalyx underlying mucus transport over mucosal surfaces of the airways, Mucosal Immunology, 6 (2013) 379-392.
[10] B. Button, L.H. Cai, C. Ehre, M. Kesimer, D.B. Hill, J.K. Sheehan, R.C. Boucher, M. Rubinstein, A Periciliary Brush Promotes the Lung Health by Separating the Mucus Layer from Airway Epithelia, Science, 337 (2012) 937-941.
[11] C. Taylor, A. Allen, P.W. Dettmar, J.P. Pearson, Two rheologically different gastric mucus secretions with different putative functions, Biochimica Et Biophysica Acta-General Subjects, 1674 (2004) 131-138.
[12] C. Atuma, V. Strugala, A. Allen, L. Holm, The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo, American Journal of Physiology-Gastrointestinal and Liver Physiology, 280 (2001) G922-G929.
[13] M.S. Ali, J.P. Pearson, Upper airway mucin gene expression: A review, Laryngoscope, 117 (2007) 932-938.
[14] B. Asgharian, W. Hofmann, F.J. Miller, Mucociliary clearance of insoluble particles from the tracheobronchial airways of the human lung, Journal of Aerosol Science, 32 (2001) 817-832.
[15] A.J. Bron, J.M. Tiffany, S.M. Gouveia, N. Yokoi, L.W. Voon, Functional aspects of the tear film lipid layer, Experimental Eye Research, 78 (2004) 347-360.
[16] J. Bridges, U.K. Kenneth Dawson, I.R.L. Wim de Jong, Rivm, T. Jung, A. Proykova, Q. Chaudhry, Fera, U. K., R. Duncan, Cardiff, E. Gaffet, Cnrs, K. Jensen, Nrcwe, W. Kreyling, H. Zentrum München, D. Bernadette Quinn, Echa, F. I., Scientific Committee on Emerging and Newly Identified Health Risks SCENIHR: Scientific Basis for the Definition of the Term “Nanomaterial”, 2010.
[17] J.P. Pearson, P.I. Chater, M.D. Wilcox, The properties of the mucus barrier, a unique gel - how can nanoparticles cross it?, Therapeutic Delivery, 7 (2016) 229-244.
[18] L.M. Ensign, A. Henning, C.S. Schneider, K. Maisel, Y.Y. Wang, M.D. Porosoff, R. Cone, J. Hanes, Ex Vivo Characterization of Particle Transport in Mucus Secretions Coating Freshly Excised Mucosal Tissues, Molecular Pharmaceutics, 10 (2013) 2176-2182.
[19] L.M. Ensign, B.C. Tang, Y.Y. Wang, T.A. Tse, T. Hoen, R. Cone, J. Hanes, Mucus-Penetrating Nanoparticles for Vaginal Drug Delivery Protect Against Herpes Simplex Virus, Science Translational Medicine, 4 (2012) 1-10.

[20] M. Abdulkarim, N. Agullo, B. Cattoz, P. Griffiths, A. Bernkop-Schnurch, S. Borros, M. Gumbleton, Nanoparticle diffusion within intestinal mucus: Three-dimensional response analysis dissecting the impact of particle surface charge, size and heterogeneity across polyelectrolyte, pegylated and viral particles, European Journal of Pharmaceutics and Biopharmaceutics, 97 (2015) 230-238.
[21] F. Laffleur, F. Hintzen, G. Shahnaz, D. Rahmat, K. Leithner, A. Bernkop-Schnurch, Development and in vitro evaluation of slippery nanoparticles for enhanced diffusion through native mucus, Nanomedicine, 9 (2014) 387-396.
[22] S. Dunnhaupt, O. Kammona, C. Waldner, C. Kiparissides, A. Bernkop-Schnurch, Nano-carrier systems: Strategies to overcome the mucus gel barrier, European Journal of Pharmaceutics and Biopharmaceutics, 96 (2015) 447-453.
[23] M.W. Joraholmen, P. Basnet, G. Acharya, N. Salko-Basnet, PEGylated liposomes for topical vaginal therapy improve delivery of interferon alpha, European Journal of Pharmaceutics and Biopharmaceutics, 113 (2017) 132-139.
[24] T. Karamanidou, K. Karidi, V. Bourganis, K. Kontonikola, O. Kammona, C. Kiparissides, Effective incorporation of insulin in mucus permeating self-nanoemulsifying drug delivery systems, European Journal of Pharmaceutics and Biopharmaceutics, 97 (2015) 223-229.
[25] O. Zupancic, A. Partenhauser, H.T. Lam, J. Rohrer, A. Bernkop-Schnurch, Development and in vitro characterisation of an oral self-emulsifying delivery system for daptomycin, European Journal of Pharmaceutical Sciences, 81 (2016) 129-136.
[26] S. Takatsuka, T. Morita, A. Koguchi, Y. Horikiri, H. Yamahara, H. Yoshino, Synergistic absorption enhancement of salmon calcitonin and reversible mucosal injury by applying a mucolytic agent and a non-ionic surfactant, International Journal of Pharmaceutics, 316 (2006) 124-130.
[27] C. Muller, G. Perera, V. Konig, A. Bernkop-Schnurch, Development and in vivo evaluation of papain-functionalized nanoparticles, European Journal of Pharmaceutics and Biopharmaceutics, 87 (2014) 125-131.
[28] J. Pearson, A. Allen, C. Venables, Gastric mucus - isolation and polymeric structure of the undegraded glycoprotein - its breakdown by pepsin, Gastroenterology, 78 (1980) 709-715.
[29] G. Hetenyi, J. Griesser, I. Nardin, A. Bernkop-Schnurch, Combination of SEDDS and Preactivated Thiomer Technology: Incorporation of a Preactivated Thiolated Amphiphilic Polymer into Self-Emulsifying Delivery Systems, Pharmaceutical research, 34 (2017) 1171-1179.
[30] S. Bonengel, F. Prufert, G. Perera, J. Schauer, A. Bernkop-Schnurch, Polyethylene imine-6-phosphogluconic acid nanoparticles - a novel zeta potential changing system, International Journal of Pharmaceutics, 483 (2015) 19-25.
[31] W. Suchaoin, I.P. de Sousa, K. Netsomboon, H.T. Lam, F. Laffleur, A. Bernkop-Schnurch, Development and in vitro evaluation of zeta potential changing self-emulsifying drug delivery systems for enhanced mucus permeation, International Journal of Pharmaceutics, 510 (2016) 255-262.
[32] V.J. Broughton-Head, J.R. Smith, J. Shur, J.K. Shute, Actin limits enhancement of nanoparticle diffusion through cystic fibrosis sputum by mucolytics, Pulmonary Pharmacology & Therapeutics, 20 (2007) 708-717.
[33] J. Deacon, S.M. Abdelghany, D.J. Quinn, D. Schmid, J. Megaw, R.F. Donnelly, D.S. Jones, A. Kissenpfennig, J.S. Elborn, B.F. Gilmore, C.C. Taggart, C.J. Scott, Antimicrobial efficacy of tobramycin polymeric nanoparticles for Pseudomonas aeruginosa infections in cystic fibrosis: Formulation, characterisation and functionalisation with dornase alfa (DNase), Journal of Controlled Release, 198 (2015) 55-61.
[34] J.M. Bailey, New mechanisms for effects of antiinflammatory glucocorticoids, Biofactors, 3 (1991) 97-102.
[35] M. Copeman, J. Matuz, A.J. Leonard, J.P. Pearson, P.W. Dettmar, A. Allen, The gastroduodenal mucus barrier and its role in protection against luminal pepsins - the effect of 16,16-dimethyl prostaglandin-e(2), carbopol-polyacrylate, sucralfate and bismuth subsalicylate, Journal of Gastroenterology and Hepatology, 9 (1994) S55-S59.

[36] H. Suleyman, A. Albayrak, M. Bilici, E. Cadirci, Z. Halici, Different Mechanisms in Formation and Prevention of Indomethacin-induced Gastric Ulcers, Inflammation, 33 (2010) 224-234.
[37] Y.Y. Wang, S.K. Lai, C. So, C. Schneider, R. Cone, J. Hanes, Mucoadhesive Nanoparticles May Disrupt the Protective Human Mucus Barrier by Altering Its Microstructure, Plos One, 6 (2011).
[38] A. Sharma, K. Jyoti, V. Bansal, U.K. Jain, B. Bhushan, J. Madan, Soluble telmisartan bearing poly (ethylene glycol) conjugated chitosan nanoparticles augmented drug delivery, cytotoxicity, apoptosis and cellular uptake in human cervical cancer cells, Mater. Sci. Eng. C-Mater. Biol. Appl., 72 (2017) 69-76.
[39] J. Todoroff, R. Vanbever, Fate of nanomedicines in the lungs, Current Opinion in Colloid & Interface Science, 16 (2011) 246-254.
[40] Y.Y. Wang, S.K. Lai, J.S. Suk, A. Pace, R. Cone, J. Hanes, Addressing the PEG Mucoadhesivity Paradox to Engineer Nanoparticles that "Slip" through the Human Mucus Barrier, Angewandte Chemie-International Edition, 47 (2008) 9726-9729.
[41] S.S. Olmsted, J.L. Padgett, A.I. Yudin, K.J. Whaley, T.R. Moench, R.A. Cone, Diffusion of macromolecules and virus-like particles in human cervical mucus, Biophysical Journal, 81 (2001) 1930-1937.
[42] J.A. Redman, S.B. Grant, T.M. Olson, M.E. Hardy, M.K. Estes, Filtration of recombinant Norwalk virus particles and bacteriophage MS2 in quartz sand: Importance of electrostatic interactions, Environmental Science & Technology, 31 (1997) 3378-3383.
[43] I. Samandoulgou, I. Fliss, J. Jean, Zeta Potential and Aggregation of Virus-Like Particle of Human Norovirus and Feline Calicivirus Under Different Physicochemical Conditions, Food and Environmental Virology, 7 (2015) 249-260.
[44] S. Di Gioia, A. Trapani, S. Castellani, A. Carbone, G. Belgiovine, E.F. Craparo, G. Puglisi, G. Cavallaro, G. Trapani, M. Conese, Nanocomplexes for gene therapy of respiratory diseases: Targeting and overcoming the mucus barrier, Pulmonary Pharmacology & Therapeutics, 34 (2015) 8-24.
[45] S.Y. Wu, H.I. Chang, M. Burgess, N.A.J. McMillan, Vaginal delivery of siRNA using a novel PEGylated lipoplex-entrapped alginate scaffold system, Journal of Controlled Release, 155 (2011) 418-426.
[46] H.P. Li, M.L. Chen, Z.G. Su, M.J. Sun, Q.N. Ping, Size-exclusive effect of nanostructured lipid carriers on oral drug delivery, International Journal of Pharmaceutics, 511 (2016) 524-537.
[47] A.C. Groo, F. Lagarce, Mucus models to evaluate nanomedicines for diffusion, Drug Discovery Today, 19 (2014) 1097-1108.
[48] R.A. Cone, Barrier properties of mucus, Advanced Drug Delivery Reviews, 61 (2009) 75-85.
[49] E. Samaridou, K. Karidi, I.P. de Sousa, B. Cattoz, P. Griffiths, O. Kammona, A. Bernkop-Schnürch, C. Kiparissides, Enzyme-Functionalized PLGA Nanoparticles with Enhanced Mucus Permeation Rate, Nano LIFE, 04 (2014) 1441013.
[50] I.P. de Sousa, B. Cattoz, M.D. Wilcox, P.C. Griffiths, R. Dalgliesh, S. Rogers, A. Bernkop-Schnurch, Nanoparticles decorated with proteolytic enzymes, a promising strategy to overcome the mucus barrier, European Journal of Pharmaceutics and Biopharmaceutics, 97 (2015) 257-264.
[51] I.P. de Sousa, C. Steiner, M. Schmutzler, M.D. Wilcox, G.J. Veldhuis, J.P. Pearson, C.W. Huck, W. Salvenmoser, A. Bernkop-Schnurch, Mucus permeating carriers: formulation and characterization of highly densely charged nanoparticles, European Journal of Pharmaceutics and Biopharmaceutics, 97 (2015) 273-279.
[52] M.C. Rose, MUCUS A2 - Laurent, Geoffrey J, in: S.D. Shapiro (Ed.) Encyclopedia of Respiratory Medicine, Academic Press, Oxford, 2006, pp. 62-66.
[53] F.J.J. Fogg, D.A. Hutton, K. Jumel, J.P. Pearson, S.E. Harding, A. Allen, Characterization of pig colonic mucins, Biochemical Journal, 316 (1996) 937-942.
[54] S. Kollner, S. Dunnhaupt, C. Waldner, S. Hauptstein, I.P. de Sousa, A. Bernkop-Schnurch, Mucus permeating thiomer nanoparticles, European Journal of Pharmaceutics and Biopharmaceutics, 97 (2015) 265-272.

[55] C.R. Miller, B. Bondurant, S.D. McLean, K.A. McGovern, D.F. O'Brien, Liposome-cell interactions in vitro: Effect of liposome surface charge on the binding and endocytosis of conventional and sterically stabilized liposomes, Biochemistry, 37 (1998) 12875-12883.
[56] Y.J. Ma, R.J.M. Nolte, J. Cornelissen, Virus-based nanocarriers for drug delivery, Advanced Drug Delivery Reviews, 64 (2012) 811-825.
[57] C.R. Kaiser, M.L. Flenniken, E. Gillitzer, A.L. Harmsen, A.G. Harmsen, M.A. Jutila, T. Douglas, M.J. Young, Biodistribution studies of protein cage nanoparticles demonstrate broad tissue distribution and rapid clearance in vivo, International Journal of Nanomedicine, 2 (2007) 715-733.
[58] P. Singh, D. Prasuhn, R.M. Yeh, G. Destito, C.S. Rae, K. Osborn, M.G. Finn, M. Manchester, Bio-distribution, toxicity and pathology of cowpea mosaic virus nanoparticles in vivo, Journal of Controlled Release, 120 (2007) 41-50.
[59] M.G. Smirnova, J.P. Birchall, J.P. Pearson, TNF-alpha in the regulation of MUC5AC secretion: Some aspects of cytokine-induced mucin hypersecretion on the in vitro model, Cytokine, 12 (2000) 1732-1736.
[60] X. Lin, J. Xie, G. Niu, F. Zhang, H.K. Gao, M. Yang, Q.M. Quan, M.A. Aronova, G.F. Zhang, S. Lee, R. Leaprnan, X.Y. Chen, Chimeric Ferritin Nanocages for Multiple Function Loading and Multimodal Imaging, Nano Lett., 11 (2011) 814-819.
[61] S. Kukulj, M. Jaganjac, M. Boranic, S. Krizanac, Z. Santic, M. Poljak-Blazi, Altered iron metabolism, inflammation, transferrin receptors, and ferritin expression in non-small-cell lung cancer, Med. Oncol., 27 (2010) 268-277.
[62] X.L. Huang, J. Chisholm, J. Zhuang, Y.Y. Xiao, G. Duncan, X.Y. Chen, J.S. Suk, J. Hanes, Protein nanocages that penetrate airway mucus and tumor tissue, Proc. Natl. Acad. Sci. U. S. A., 114 (2017) E6595-E6602.
[63] H. Malhaire, J.C. Gimel, E. Roger, J.P. Benoit, F. Lagarce, How to design the surface of peptide-loaded nanoparticles for efficient oral bioavailability?, Advanced Drug Delivery Reviews, 106 (2016) 320-336.
[64] C.A. Vasconcellos, P.G. Allen, M.E. Wohl, J.M. Drazen, P.A. Janmey, T.P. Stossel, Reduction in viscosity of cystic-fibrosis sputum in-vitro by gelsolin, Science, 263 (1994) 969-971.
[65] L.W. Matthews, S. Spector, J. Lemm, J.L. Potter, Studies on pulmonary secretions. I. The over-all chemical composition of pulmonary secretions from patients with cystic fibrosis, bronchiectasis, and laryngectomy, The American review of respiratory disease, 88 (1963) 199-204.
[66] J.B. Lyczak, C.L. Cannon, G.B. Pier, Lung infections associated with cystic fibrosis, Clinical Microbiology Reviews, 15 (2002) 194-222.
[67] A. Chuchalin, E. Amelina, F. Bianco, Tobramycin for inhalation in cystic fibrosis: Beyond respiratory improvements, Pulmonary Pharmacology & Therapeutics, 22 (2009) 526-532.
[68] R. Balsamo, L. Lanata, C.G. Egan, Mucoactive drugs, European respiratory review : an official journal of the European Respiratory Society, 19 (2010) 127-133.
[69] K.K. Kumar, S. Karnati, M.B. Reddy, R. Chandramouli, Caco-2 cell lines in drug discovery- an updated perspective, Journal of basic and clinical pharmacy, 1 (2010) 63-69.
[70] M. Minekus, M. Alminger, P. Alvito, S. Ballance, T. Bohn, C. Bourlieu, F. Carriere, R. Boutrou, M. Corredig, D. Dupont, C. Dufour, L. Egger, M. Golding, S. Karakaya, B. Kirkhus, S. Le Feunteun, U. Lesmes, A. Macierzanka, A. Mackie, S. Marze, D.J. McClements, O. Menard, I. Recio, C.N. Santos, R.P. Singh, G.E. Vegarud, M.S.J. Wickham, W. Weitschies, A. Brodkorb, A standardised static in vitro digestion method suitable for food - an international consensus, Food & Function, 5 (2014) 1113-1124.
[71] M. Boegh, M. García-Díaz, A. Müllertz, H.M. Nielsen, Steric and interactive barrier properties of intestinal mucus elucidated by particle diffusion and peptide permeation, European Journal of Pharmaceutics and Biopharmaceutics, 95 (2015) 136-143.
[72] G.J. Mahler, M.L. Shuler, R.P. Glahn, Characterization of Caco-2 and HT29-MTX cocultures in an in vitro digestion/cell culture model used to predict iron bioavailability, The Journal of Nutritional Biochemistry, 20 (2009) 494-502.

[73] M. Minekus, The TNO Gastro-Intestinal Model (TIM), in: K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, H. Wichers (Eds.) The Impact of Food Bioactives on Health: in vitro and ex vivo models, Springer International Publishing, Cham, 2015, pp. 37-46.
[74] H. Friedl, S. Dunnhaupt, F. Hintzen, C. Waldner, S. Parikh, J.P. Pearson, M.D. Wilcox, A. Bernkop-Schnurch, Development and Evaluation of a Novel Mucus Diffusion Test System Approved by Self-Nanoemulsifying Drug Delivery Systems, Journal of Pharmaceutical Sciences, 102 (2013) 4406-4413.
[75] P. Van den Abbeele, C. Grootaert, S. Possemiers, W. Verstraete, K. Verbeken, T. Van de Wiele, In vitro model to study the modulation of the mucin-adhered bacterial community, Applied Microbiology and Biotechnology, 83 (2009) 349-359.
[76] M. Marzorati, B. Vanhoecke, T. De Ryck, M. Sadaghian Sadabad, I. Pinheiro, S. Possemiers, P. Van den Abbeele, L. Derycke, M. Bracke, J. Pieters, T. Hennebel, H.J. Harmsen, W. Verstraete, T. Van de Wiele, The HMI™ module: a new tool to study the Host-Microbiota Interaction in the human gastrointestinal tract in vitro, BMC Microbiology, 14 (2014) 133.
[77] P. Georgiades, P.D. Pudney, D.J. Thornton, T.A. Waigh, Particle tracking microrheology of purified gastrointestinal mucins, Biopolymers, 101 (2014) 366-377.
[78] M. Marzorati, S. Possemiers, P. Van Den Abbeele, T. Van De Wiele, B. Vanhoecke, W. Verstraete, Technology and method to study microbial growth and adhesion to host-related surfaces and the host-microbiota interaction, Universiteit Gent, 2009.
[79] A.G. Oomen, A. Hack, M. Minekus, E. Zeijdner, C. Cornelis, G. Schoeters, W. Verstraete, T. Van de Wiele, J. Wragg, C.J.M. Rompelberg, A.J.A.M. Sips, J.H. Van Wijnen, Comparison of Five In Vitro Digestion Models To Study the Bioaccessibility of Soil Contaminants, Environmental Science & Technology, 36 (2002) 3326-3334.
[80] W.M. Faulks Richard, Dynamic gastric model, Plant Bioscience Limited, 2012.
[81] I. Mainville, Y. Arcand, E.R. Farnworth, A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics, International Journal of Food Microbiology, 99 (2005) 287-296.
[82] S.R. Kong F, A human gastric simulator (HGS) to study food digestion in human stomach., Journal of Food Science, 75 (2010) 627-635.
[83] P.M. Reis, T.W. Raab, J.Y. Chuat, M.E. Leser, R. Miller, H.J. Watzke, K. Holmberg, Influence of Surfactants on Lipase Fat Digestion in a Model Gastro-intestinal System, Food biophysics, 3 (2008) 370-381.
[84] K. Molly, M.V. Woestyne, I. De Smet, W. Verstraete, Validation of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) Reactor Using Microorganism-associated Activities, Microbial Ecology in Health and Disease, 7 (1994) 191-200.
[85] M.S.-P. Minekus, M.; Bernalier, A.; Marol-Bonnin, S.; Havenaar, R.; Marteau, P.; Alric, M.; Fonty, G.; Huis in't Veld, J. H. J., A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products, Applied Microbiology & Biotechnology, 53 (2000) 108.
[86] Z.E. Blanquet S, Beyssac E, Meunier JP, Denis S, Havenaar R, Alric M., A dynamic artificial gastrointestinal system for studying the behavior of orally administered drug dosage forms under various physiological conditions., Pharmaceutical research, 21 (2004) 585-591.
[87] Wickham M, Faulks R, Mills C, In vitro digestion methods for assessing the effect of food structure on allergen breakdown., Molecular Nutrition & Food Research, 53 (2009) 952-958.
[88] Blanquet S, Zeijdner E, Beyssac E, Meunier JP, Denis S, Havenaar R, A. M., A dynamic artificial gastrointestinal system for studying the behavior of orally administered drug dosage forms under various physiological conditions, Pharmaceutical research, 21 (2004) 585-591.
[89] E. Frohlich, The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles, International Journal of Nanomedicine, 7 (2012) 5577-5591.

[90] X. Luo, M. Feng, S.R. Pan, Y.T. Wen, W. Zhang, C.B. Wu, Charge shielding effects on gene delivery of polyethylenimine/DNA complexes: PEGylation and phospholipid coating, Journal of Materials Science-Materials in Medicine, 23 (2012) 1685-1695.
[91] C. Fruijtier-Polloth, Safety assessment on polyethylene glycols (PEGs) and their derivatives as used in cosmetic products, Toxicology, 214 (2005) 1-38.
[92] A.K. Jain, N.K. Swarnakar, C. Godugu, R.P. Singh, S. Jain, The effect of the oral administration of polymeric nanoparticles on the efficacy and toxicity of tamoxifen, Biomaterials, 32 (2011) 503-515.
[93] N.M. Zaki, M.M. Hafez, Enhanced Antibacterial Effect of Ceftriaxone Sodium-Loaded Chitosan Nanoparticles Against Intracellular Salmonella typhimurium, Aaps Pharmscitech, 13 (2012) 411-421.
[94] Z. Ahmad, R. Pandey, S. Sharma, G.K. Khuller, Novel chemotherapy for tuberculosis: chemotherapeutic potential of econazole- and moxifloxacin-loaded PLG nanoparticles, International Journal of Antimicrobial Agents, 31 (2008) 142-146.
[95] R. Landsiedel, E. Fabian, L. Ma-Hock, W. Wohlleben, K. Wiench, F. Oesch, B. van Ravenzwaay, Toxico-/biokinetics of nanomaterials, Archives of Toxicology, 86 (2012) 1021-1060.
[96] T. Iglesias, A.L. de Cerain, J.M. Irache, N. Martin-Arbella, M. Wilcox, J. Pearson, A. Azqueta, Evaluation of the cytotoxicity, genotoxicity and mucus permeation capacity of several surface modified poly(anhydride) nanoparticles designed for oral drug delivery, International Journal of Pharmaceutics, 517 (2017) 67-79.
[97] A.R. Collins, A. Azqueta, Single-Cell Gel Electrophoresis Combined with Lesion-Specific Enzymes to Measure Oxidative Damage to DNA, in: P.M. Conn (Ed.) Laboratory Methods in Cell Biology: Biochemistry and Cell Culture, Elsevier Academic Press Inc, San Diego, 2012, pp. 69-92.
[98] S.J. Xiong, S. George, H.Y. Yu, R. Damoiseaux, B. France, K.W. Ng, J.S.C. Loo, Size influences the cytotoxicity of poly (lactic-co-glycolic acid) (PLGA) and titanium dioxide (TiO2) nanoparticles, Archives of Toxicology, 87 (2013) 1075-1086.
[99] S. Mura, H. Hillaireau, J. Nicolas, B. Le Droumaguet, C. Gueutin, S. Zanna, N. Tsapis, E. Fattal, Influence of surface charge on the potential toxicity of PLGA nanoparticles towards Calu-3 cells, International Journal of Nanomedicine, 6 (2011) 2591-2605.
[100] T. Mosmann, Rapid colorimetric assay for cellular growth and survival - application to proliferation and cyto-toxicity assays, Journal of Immunological Methods, 65 (1983) 55-63.
[101] V. Stone, H. Johnston, R.P.F. Schins, Development of in vitro systems for nanotoxicology: methodological considerations, Critical Reviews in Toxicology, 39 (2009) 613-626.
[102] M.D. Wilcox, L.K. Van Rooij, P.I. Chater, I.P. de Sousa, J.P. Pearson, The effect of nanoparticle permeation on the bulk rheological properties of mucus from the small intestine, European Journal of Pharmaceutics and Biopharmaceutics, 96 (2015) 484-487.
[103] A.E. Bell, L.A. Sellers, A. Allen, W.J. Cunliffe, E.R. Morris, S.B. Rossmurphy, Properties of gastric and duodenal mucus - effect of proteolysis, disulfide reduction, bile, acid, ethanol, and hypertonicity on mucus gel structure, Gastroenterology, 88 (1985) 269-280.