# TRANSEPITHELIAL TRANSPORT OF PAMAM DENDRIMERS

# ACROSS ISOLATED INTESTINAL TISSUE

by

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# STATEMENT OF DISSERTATION APPROVAL

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# ABSTRACT

Poly(amido amine) (PAMAM) dendrimers have shown potential to carry poorly absorbed drugs across the intestinal barrier and into systemic circulation, reducing the need for intravenous injections. Much of the in vitro transepithelial transport of PAMAM dendrimers to date has been investigated using Caco-2 monolayers which lack the microvilli morphology and enzymes present in isolated intestinal tissues. In addition, a challenge in predicting oral absorption is establishing a correlation between transport across rodent and human intestinal tissues. This dissertation focused on investigating the transepithelial transport of PAMAM dendrimers across rat and human isolated intestinal tissues. Permeability values in isolated tissues were compared with those across Caco-2 cell monolayers. Results indicate a difference in transport of PAMAM dendrimers, morphological changes and transepithelial electrical resistance between Caco-2 cell monolayers, rat and human intestinal tissue models. A relatively high transport rate across the tissues, given the macromolecular nature of PAMAM dendrimers, shows promise for use of these constructs for oral delivery in human.

To my God

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# ABBREVIATIONS

| | |
|---|---|
| ∆Isc | Short circuit current |
| AAC | Area above the curve |
| AB | Apical to basolateral |
| ANOVA | One-way analysis of variance |
| ATRA | All-trans retinoic acid |
| AUC | Area under the curve |
| BA | Basolateral to apical |
| BCA | Poly(butyl cyanoacrylate) |
| BCS | Biopharmaceutical classification system |
| Boc | Tert-butyl |
| BSA | Bovine serum albumin |
| $C_{max}$ | Peak plasma concentration |
| CMC | Critical micelle concentration |
| CMT | Critical micelle temperature |
| CSK | CSKSSDYQC targeting peptide |
| DI | Deionized |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMF | Dimethylformamide |
| DOPE | Dioleoylphosphatidylethanolamine |

| | |
|---|---|
| DPPC | Dipalmitoylphosphatidylcholine |
| DTPA | Diethylene triamine pentaacetic acid |
| ED | Ethylenediamine |
| EDC | N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide |
| EDTA | Ethylenediaminetetraacetic acid |
| EI | Endocytic index |
| EPR | Enhanced permeability and retention |
| F | Bioavailability |
| FBS | Fetal bovine serum |
| FD10 | 10 kDa FITC-dextran |
| FD4 | 4 kDa FITC-dextran |
| FITC | Fluorescein isothiocyanate |
| FMOC | Fluorenylmethyloxycarbonyl |
| FPLC | Fast protein liquid chromatography |
| G0.5 | Generation 0.5 |
| G1.0 | Generation 1 |
| G1.5 | Generation 1.5 |
| G2.0 | Generation 2 |
| G3.5 | Generation 3.5 |
| G4.0 | Generation 4 |
| G6.5 | Generation 6.5 |
| GI | Gastrointestinal |
| H&E | Hematoxylin and eosin |

| | |
|---|---|
| HBSS | Hank’s balanced salt solution |
| HBTU | O-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate |
| HEPES | (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) |
| HM-HPC | Poly(oxy ethylene cetylether hydroxyl propylcellulose) |
| HPMA | *N*-2-(hydroxypropyl)methacrylamide |
| IACUC | Institutional Animal Care and Use Committee |
| IRB | Institutional review board |
| KH | Krebs-Hensleit |
| $LD_{50}$ | Lethal dose 50 |
| LDH | Lactate dehydrogenase |
| MALDI-TOF | Matrix assisted laser desorption ionization time of flight mass spectrometry |
| MLC | Myosin light chain |
| MLCP | Myosin light chain phosphatase |
| mme$PEG_{750}$P(CL-co-TMC | Monomethyletherpoly(oxyethylene $glycol_{750}$)-poly(caprolactone-co-trimethylene carbonate |
| MTD | Maximum tolerated dose |
| MTT | (3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide) |

| | |
|---|---|
| MW | Molecular weight |
| NMM | N-methylmorpholine |
| NMR | Nuclear magnetic resonance |
| PAMAM | Poly(amido amine) |
| PAMPA | Parallel artificial membrane permeability assay |
| $P_{app}$ | Apparent permeability |
| PBS | Phosphate buffered saline |
| PCA | Poly(Alkyl Cyanoacrylates) |
| PD | Potential difference |
| $P_{eff}$ | Effective permeability |
| PEG | Poly(ethylene glycol) |
| PEG-b-P(VBODENA) | Poly(ethylene glycol) poly(2-(4-vinylbenzyloxy)-N,N-diethylnicotinamide) |
| PEI | Poly(ethyleneimine) |
| PEP | Peptide penetration enhancers |
| PEP | Poly(ethylene oxide) |
| PGA | Poly(glycolic acid) |
| P-gp | P-glycoprotein |
| PLA | Poly(lactic acid) |
| PLGA | Poly(lactic-*co*-glycolic acid) |
| PO | Poly(propylene oxide) |
| SEC | Size exclusion chromatography |
| SN-38 | 7-ethyl-10-hydroxy-camptothecin |
| Tagat S | PEG-30 glyceryl stearate |

| | |
|---|---|
| TEER | Transepithelial electrical resistance |
| TEM | Transmission electron microscopy |
| THAP | 2′,4′,6′-trihydroxyacetophenone monohydrate |
| $t_{max}$ | Time to maximum plasma concentration |
| TPGS | D-α-tocopheryl poly(ethylene glycol) succinate |
| UCD | University College Dublin |
| UEA-1 | Ulex europaeus 1 |
| v/v | Volume per volume |
| w/v | Weight per volume |
| WST-1 | 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2h-5-tetrazolio]-1,3-benzene disulfonate) |

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# CHAPTER 1

# INTRODUCTION

## 1.1 Oral drug delivery

Carrier based drug delivery has been used to improve the pharmacokinetics and biodistribution of small molecule drugs.[1] Polymeric carriers particularly have been used to enhanced drug uptake at the site of action and minimize off-target effects.[2] Water soluble polymer-based drug delivery can improve the safety of therapeutically active compounds with intrinsically poor water solubility and high toxicity.[3] Nanomedicines such as polymers and liposomes have shown promise in altering the intracellular accumulation and oral permeability of drugs.[4,5] Important targeting agents such as antibodies and peptides can be conjugated to delivery vehicles making such carriers useful for targeting sites of disease.[6]

Many anticancer therapies are limited by poor water solubility and dose-limiting toxicity.[7] Conjugation of chemotherapeutics to macromolecular water soluble carriers such as poly(ethylene glycol) and *N*-(2-hydroxyproply)methacrylamide (HPMA) copolymers can improve their biodistribution by increasing the drug concentration in cancerous tissue due to enhanced permeability and retention effect.[8] Increased tumor accumulation and

reduced offsite release of the drug are basis for polymer utility in drug delivery.[9]

Additionally, many chemotherapeutics have low water solubility and poor oral absorption limiting their delivery by the intravenous route.[10] This requires the patient to be present at the hospital for recurring visits, with significant direct and indirect costs.[11] Attachment of poorly soluble drugs to water soluble polymers can enhance solubility, but the macromolecular structure of most polymers limits dosing to intravenous routes.[12] Strong patient preference for oral formulations and the significant advantages of polymer therapeutics provide rationale for development of oral polymeric drug delivery systems.[10] An oral polymer therapeutic has the combined advantages of increased water solubility, enhanced delivery to the site of action, a more flexible dosing regimen and reduced need for hospital procedures.[13]

Oral drug delivery is challenging due to the harsh chemical and enzymatic environment of the intestinal tract.[14] The human gastrointestinal tract is efficiently designed to limit the absorption of macromolecules.[14,15] Intestinal gastric pH can reach levels as low as one, while proteolytic enzymes (such as trypsin) and metabolic enzymes (such as cytochrome P450's) continuously degrade functional pharmaceuticals into kidney clearable content, not to mention efflux proteins, bile salts and food bolus which can hinder or alter pharmaceutical absorption.[16–18] Therapies designed for oral delivery must pass through the low pH of the stomach, withstand various enzymes released into the chyme and localized in the brush border, and be able to penetrate the epithelial barrier of the gut.

Poly(amido amine) (PAMAM) dendrimers have been studied for the oral delivery of poorly absorbed compounds.[4] PAMAM dendrimers are a class of hyperbranched water soluble carriers that have shown potential in increasing the absorption of both conjugated and entrapped drugs.[19,20] These dendrimers are synthesized through the step-wise addition of ethylene diamine and methyl acrylate forming repeating layers termed generations.[21] Each subsequent generation increases in diameter linearly, but increases in number of surface groups exponentially.[22] Thus a generation 4 (G4) dendrimer has 64 surface groups and a 4nm diameter while a G5 dendrimer has 128 surface groups and a 5nm diameter. These surface groups can be modified with positively charged amine groups, neutral hydroxyl or negatively charged carboxyl groups.[23] In addition, various drugs targeting ligands and imaging agents can be attached to dendrimers making them a potential multifunctional drug delivery vehicle.[24]

Many studies of PAMAM dendrimer intestinal penetration have been performed to date. In most of these studies, Caco-2 cell models were used to evaluate permeability and epithelial toxicity of the dendrimers.[20,25–32] Dendrimer permeation across Caco-2 is a function of the generation, concentration, and incubation time.[27] Mechanistic studies into the routes via which PAMAM dendrimers can penetrate the intestinal epithelium show that specific pharmacologic endocytosis inhibitors reduced the flux of G3.5 PAMAM dendrimers across Caco-2 monolayers.[26] Other work showed that PAMAM dendrimer transport across Caco-2 cells was clathrin, dynamin and energy-dependent.[33] These studies showed that dendrimers facilitated tight junction

opening, as evidenced by occludin staining and increased mannitol transport.[29–31,33,34] Dendrimer permeability across Caco-2 monolayers appears to be via a combination of the transcellular and paracellular route. These studies have provided an initial understanding of dendrimer intestinal transport, but have limitations of Caco-2 cells such as the lack of mucous layers, intestinal morphology and in vivo enzyme levels, present in the human intestinal tract.[35–38]

Indeed the discrepancy between permeability results in vivo and Caco-2 monolayers has become more apparent with further research.[19,29,39] Previously it was noted that concentrations as low as 0.1mM of amine terminated G4 PAMAM dendrimers caused a reduction in transepithelial electrical resistance (TEER) and increased mannitol transport across Caco-2 monolayers.[29] On the other hand, permeability data observed in CD-1 mice indicated no increase in mannitol permeability or tight junction opening when PAMAM dendrimers were administered orally at concentrations of ~2mM and ~7mM for G4-$NH_2$ and G3.5-COOH, respectively.[19] This concentration is almost 100 fold higher than “toxic” Caco-2 concentrations and still no significant changes in epithelial morphology or mannitol permeability have been noted. These discrepancies have led us to evaluate the permeability of dendrimers across isolated intestinal tissue models using the Ussing the chamber technique.

The rationale for the use of isolated tissue models is that they provide higher fidelity of permeability results to human absorption than other models (i.e., Caco-2, parallel artificial membrane permeability assay (PAMPA), etc.).[40] PAMPA assays lack enzymes and cellular functions including lipid bilayers and

drug absorption. Caco-2 cells have additional limitations including lack of intestinal enzyme levels and mucous layers.[41] In vivo studies in animals are feasible, but doses can be diluted in the gastrointestinal tract, causing concentrations to vary and making transepithelial transport difficult to quantify. Also, in vivo results generally include first pass metabolism and tissue distribution that make transepithelial transport difficult to quantify. Rationale for the use of isolated intestinal tissue using the Ussing chambers includes the ability to control concentration, which can be variable in vivo due to dilution of intestinal contents. Also an advantage of Ussing chambers is the ability to reduce the quantity of animals required for experiment compared to in vivo studies. Finally, isolated tissue models have a higher ability to predict human absorption especially when using human tissues.[40,42] Isolated intestinal tissue in Ussing chambers provides an alternative model that can be used to evaluate the potential of PAMAM dendrimers for oral drug delivery.

Because most macromolecules have low permeability through the intestinal tract, additional penetration enhancers have been used to aid in their transport. Specifically peptide penetration enhancers (PEP) have been hypothesized to aid in the penetration of PAMAM dendrimers across the intestinal barrier.[43] The benefit of PEP over other penetration enhancers is their ability to specifically modulate an opening of the tight junctions in the intestine, without causing toxicity to the epithelial tissue.[44–47] C10 fatty acids, commonly used as penetration enhancers to enhance permeability cause significant sloughing of the epithelial surface cells.[48] PEP has shown not to cause increase

in lactate dehydrogenase (LDH) release and histological damage and simultaneously modulates the opening of tight junctions for oral drug delivery.[43] A one-month subchronic intranasal dosing of penetration enhancing peptides to rats showed no significant elevation of IL-1α or TNF-α and no damage to epithelial membranes.[49] Likewise, other arginine rich sequences showed no additional toxicity or immunogenicity in cells, in animals nor in clinical trials.[50,51] The lack of toxicity to epithelial layers and simultaneous increasing penetration of macromolecules make PEP ideal for use in conjunction with PAMAM dendrimers for oral drug delivery without inducing toxic effects.

A specific PEP with potential in macromolecular delivery is the P640 peptide which has the specific amino acid sequence of RRVEVKYDRRKKR (single letter amino acid abbreviations are used) which modulate myosin light chain phosphatases leading to an increase in activated myosin light chain and the opening of tight junctions.[51–57] The disruption of interaction of the myosin light chain phosphatase (MLCP) with myosin light chain (MLC) leads to the unregulated phosphorylation of MLC. MLC is constitutively dephosphorylated by MLCP, but when MLCP is inhibited ZIP kinase activity causes the phosphorylation of myosin light chain (MLC) to increase, leading to cellular actin-myosin contraction.[52,53] The contraction of the actin-myosin filaments in intestinal epithelial cells leads to the contraction of the perijunctional ring and the opening of tight junctions. The opening of these junctions allows drug delivery agents to pass through the paracellular space.[58] The potential of these PEPs to induce high permeability for oral drug delivery via phosphatase inhibition makes them

suitable for delivery of macromolecules such as dendrimers.[59]

## 1.2 Aims and scope of this dissertation

The **global hypothesis** of this dissertation is that investigation of PAMAM dendrimer transport through insolated intestinal tissue can predict human absorption. The **ultimate goal** is to discover if PAMAM dendrimer permeability across the intestinal tissue is sufficient for use as an oral drug delivery carrier.

This work encompasses the quantitative assessment of dendrimer permeability across isolated tissue models. Chapter 2 provides a review of the challenges of oral drug delivery with nanoparticles and the current models of intestinal absorption. The current clinical achievements of oral drug delivery using nanotechnology are summarized in this Chapter and published elsewhere.[60] Chapters 3-5 contain work adapted from previous publications.[61–63] These chapters cover research that is aimed at assessing the differences between various transepithelial transport models such as Caco-2 monolayers, rat, and human isolated intestinal tissues. Chapter 6 provides conclusions and future directions of this research. In addition, an attempt was made to assess the intestinal permeability of PAMAM dendrimers in combination with peptide penetration enhancers. The results of these studies, included in Appendix A, are inconclusive and require further investigation.

## 1.3 Specific aims

**Aim 1:** To investigate the transepithelial transport of PAMAM dendrimers across rat isolated intestinal tissue in an Ussing Chamber set up.

**Rationale**: Dendrimer transport needs to be evaluated in isolated tissue models to compare differences between Caco-2, isolated tissue and in vivo models.

**Hypothesis:** Dendrimer transport is predicted to be significantly higher than similar sized fluorescein isothiocyanate (FITC)-dextrans and free FITC controls in isolated rat intestinal epithelium. A corollary hypothesis is that 1mM concentration of dendrimers do not cause significant epithelial morphological changes.

In Chapter 3, the innate permeability of fluorescently labeled dendrimers (G3.5, G4) was assessed across rat jejunum. Histology and transepithelial electrical resistance (TEER) was examined to assess dendrimer effects on epithelial morphology and integrity.[14] C-mannitol transport was monitored to assess tight junction modulation. These studies demonstrate that PAMAM dendrimer transport is significantly higher than free FITC controls in isolated rat jejunal tissue.[61] 1mM intestinal concentrations of G3.5 and G4 dendrimer were not found to cause changes in TEER, mannitol or morphology to the isolated epithelium.

**Aim 2:** To investigate the regional transport of PAMAM dendrimers across isolated rat intestinal tissue and Caco-2 monolayers.

**Rationale:** Assessment of regional dependence of transepithelial

transport of PAMAM dendrimers aids in determining the location of maximum absorption in the intestinal tissue.

**Hypothesis:** PAMAM dendrimer intestinal transport is predicted to be greater in the jejunal region than the colonic region in isolated rat intestinal tissues.

In Chapter 4, the permeability of PAMAM dendrimers G3.5 and G4 was assessed in rat colon and jejunum using the Ussing chamber technique. Transepithelial transport across these tissues was compared with permeability across Caco-2 cell monolayers. Morphology and integrity of the epithelial barriers was investigated using histopathology, measurement of TEER and mannitol transport. Results indicate that PAMAM dendrimer permeability is greatest in isolated jejunal tissues.[62]

**Aim 3:** To investigate the transepithelial transport of PAMAM dendrimers across isolated human intestinal tissue.

**Rationale:** Isolated human tissue has a strong correlation with human fraction absorbed and thus can be used as a predictive marker for PAMAM dendrimer bioavailability in humans.

**Hypothesis**: Dendrimer permeability in human isolated intestinal tissue is predicted to be less than dendrimer permeability in rat isolated intestinal tissue.

To address this aim we assessed PAMAM dendrimer generations 3.5 and 4 regional transport in human colon and jejunum for the first time, as well as the regional permeability of mannitol in the presence of various concentrations of dendrimers. These results are discussed in Chapter 5. The concentration

dependent effect of dendrimers on human intestinal epithelial morphology was evaluated. The permeability of dendrimers between isolated human, isolated rat and Caco-2 models was investigated. Results demonstrate that dendrimer permeability is sufficient for the oral delivery of potent drugs.[63]

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# CHAPTER 2[1]

# LITERATURE REVIEW

## 2.1 Introduction

The objective of nanoparticle and polymer therapeutics is to control the spatiotemporal release and distribution of incorporated drugs to improve safety and efficacy for patients.[1] Specifically drug delivery technology can alter a drug's solubility, absorption, metabolism, elimination and biodistribution. This strategy can enhance the therapeutic index of the free drug by reducing toxicity and increasing efficacy. Additionally, many of these systems can be attached to imaging agents, drugs, and targeting ligands making them multifunctional drug delivery vehicles.[2] Examples of drug delivery carriers include nanoparticles, polymers, micelles and liposomes.[3–6]

Polymer-drug conjugates were first conceptualized in 1975 by Ringsdorf.[7] In this concept, a pharmaceutical molecule is covalently conjugated to a carrier through a degradable linker which can release the drug at the site of action. Properties of the carrier such as composition, molecular weight, architecture, surface charge and linker chemistry can be tailored to maximize efficacy and

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minimize off target release.

Nonetheless, many of these polymeric delivery systems are limited to parental injections, due to low absorption through the intestinal route (Table 2.1).[8] Oral administration of pharmaceuticals has long been the preferred route compared to parenteral injections. The benefits of the oral route include flexible dosing, higher patient compliance and reduced health care costs.[9] Many small molecules and biologicals have limited oral bioavailability due to low solubility, limited stability in the GI (gastrointestinal) tract as well as the low and variable permeation.[10,11] These challenges form the basis of the design of nanopreparations for oral delivery.[12] Oral nanopreparations have been investigated for improving the bioavailability (F) of various drugs such as heparin,[13] enalaprilat,[14] tobramycin,[15] and antitubercular drugs.[12,16,17] Nanopreparations may enhance solubility, increase stability and absorption, and control spatiotemporal release.[10] To date several nanopreparations have been approved by regulatory agencies for use in the clinic as oral dosages (e.g., Élan's Nanocrystal® technology).[18]

The long standing need for the oral delivery of proteins and peptides has been recognized.[19] Nanoparticle polymeric drug carriers have been viewed as possible formulations to improve oral bioavailability of such molecules.[20,21] For example in the case of insulin, the human small intestine mesenteric blood delivers nutrients and drugs into the hepatic portal vein which makes oral delivery of insulin more physiologically relevant given that the liver is its primary target when normally released from pancreatic beta cells.

**Table 2.1.** Examples of marketed drug delivery nanomedicines.[16,17]

| Product | Technology | Indication | Delivery Route | Company |
|---|---|---|---|---|
| Rapamune (sirolimus) | NanoCrystal Elan | immunosuppressive | oral | Pfizer/Wyeth |
| Myocet | liposomal doxorubicin | cancer—breast cancer | iv | Cephalon |
| Oncaspar | PEG-asparaginase | cancer—acute lymphocytic leukemia (ALL) | iv/im | Enzon |
| Peg-intron | PEG-interferon alpha 2b | hepatitis C | sc | Schering-Plough |
| Pegasys | PEG-interferon alpha 2a | hepatitis C | sc | Roche |
| Neulasta | PEG-hrGCSF | chemotherapy-induced neutropenia | sc | Amgen |
| Adagen | PEG-adenosine deaminase | severe combined immune deficiency syndrome | im | Enzon |
| Somavert | PEG-Visomant | acromegaly | sc | Pfizer |
| Macugen | PEG-aptanib | wet age-related macular degeneration | intravitreal | NeXstar |
| Krystexxa | PEG-loticase | chronic gout, adults refractory to conventional therapy | iv | Savient/Crealta |
| Plegridy | PEG-interferon beta-1a | relapsing multiple sclerosis | sc | Biogen |
| BEXXAR (tositumomab) | $^{131}$I-tositumomab | cancer—CD20 +ve follicular, NHL refractory to rituximab | iv | Bexxar |

Many active small molecular weight drugs have poor oral absorption and solubility.[22] Poorly soluble drugs may often have low or variable permeability across the intestinal epithelial barrier making dosing difficult to predict. Nanopreparations have been investigated for improving pharmacokinetics of poorly soluble drugs and poorly permeable drugs.[18] Compared to intravenous administration, in oral delivery a delayed time to maximum plasma concentration ($t_{max}$) and a lower peak plasma concentration ($C_{max}$) are observed. These attributes can facilitate a more flexible dosing regimen as well as decreased risk of infection from intravenous lines in chronic patients.

Oral delivery of poorly soluble and permeable drugs has been facilitated by use of penetration enhancers[23,24] and microparticles.[25–28] Permeation enhancers have been used to improve transport across the epithelial barrier, while microparticles have been used to protect drug molecules from the harsh gastrointestinal environment. Depending on composition, nanoscale structures can potentially carry out both of these important functions aiding in oral drug delivery.[20,29]

## 2.2 Physiological barriers to nanopreparations for oral delivery

Clinical success for nanopreparations has been difficult due to the inherent physiological environment of the gastrointestinal tract. The intestines are designed to break down and absorb proteins, carbohydrates and lipids into component formats.[11] Orally delivered drugs have to survive the stomach acidic

environment, the renin and pepsin enzymes, and the pancreatic enzymatic activity of the small intestine. In addition to these biological barriers, the drug has to be able to traverse across the intestinal epithelium.[11,30]

Many drug compounds are hydrophobic in nature and hence poorly soluble in aqueous environments. Many of the drugs of biopharmaceutics classification systems (BCS) class II or class IV category require significant formulation in order to enhance their solubility. Nanopreparations can be used to solvate these drugs and to create greater drug exposure to the absorption site of the targeted organ.[31,32] The dimension and shape of the delivery system can also have a significant effect on dissolution rate in the intestinal tract and subsequent oral bioavailability.[10,33–35]

An important note for oral nanopreparations is that the stomach secretions can reach a pH level as low as 1. This pH can denature or hydrolyze many exposed proteins or other acid labile linkers, thus making the stomach milieu a formidable chemical barrier to oral formulations.[11] On a related note, stomach pH can rise as high as 7 in the fed state.[36] This can influence release from enteric polymer-coated delivery systems, such as Eudragit®, which are often used in order to protect acid or pepsin susceptible materials in solid dosage forms from degrading in the stomach. Another consideration is that the pH level of the small intestine ranges from 6.6±0.5 to 7.5±0.4.[11,37]

Particle transit time varies based on region and size.[38] Other physiological factors also affect transit time. The transit time from the stomach to the duodenum depends on the type of content in the stomach and the amount of

time since previous food intake. During fasting the stomach to duodenum transit time for saline is only 12 min, but with a large meal can take as long as 4 h.[39] The carbohydrate portions usually arrive in the small intestine first while the fatty portions arrive last. Liquids can bypass the solid portions of the meal and enter the duodenum rapidly.[40] Therefore, the time for an oral nanopreparation to reach the small intestine can be drastically affected by the contents of the stomach, and the body's state of fasting. This can be critical, if for example, in cases where an oral drug is stable for only 2 h but the transit time to the small intestine takes up to 4 h. Generally, like most nutrients and drug molecules, nanopreparations are not absorbed in the stomach, but more distally in the small intestine or colon. Some nanopreparations may not even reach the absorptive surface in an intact form.

The actual flow speed of chyme through the small intestine is approximately 1-4 cm per min.[40] Bile salts help bicarbonate to neutralize acid in fluid emerging from the stomach into the duodenum, but their primary role is to emulsify fatty contents. They are actively reabsorbed via the ileal bile acid transporter and returned to the liver for reuse.[39] Bile salts can affect oral dosage forms by changing the properties of nanopreparations that are formed from micelles or surfactants. Pancreatic juice also delivers proteases, lipases, and carbohydrases, essential for further digestion in the small intestinal tract.[41] These enzymes can cause degradation or denaturation of proteins, DNA and other labels or linkers that potentially have role in stabilizing an oral nanopreparation. (see Table 2.2 for common intestinal enzymes).

**Table 2.2.** Major enzymes of the gastrointestinal tract[31]

| ***Enzyme*** | ***Site of Action*** | ***Substrate*** | ***Products of Action*** |
|---|---|---|---|
| Pepsinogen:pepsin | Stomach | Proteins | Large peptides |
| Pancreatic-amylase | Small intestine | Starch | Disaccharides |
| Trypsinogen:trypsin | Small intestine | Proteins | Large peptides |
| Chymotrypsin | Small intestine | Proteins | Large peptides |
| Elastase | Small intestine | Elastin | Large peptides |
| Carboxypeptidases | Small intestine | Large peptides | Oligopeptides |
| Aminopeptidases | Small intestine | Large peptides | Oligopeptides |
| Lipase | Small intestine | Triglycerides | Monoglycerides |
| Nucleases | Small intestine | Nucleic acids | Nucleotides |
| Enterokinase | Small intestine | Trypsinogen | Trypsin |
| Disaccharidases | Small intestine[a] | Trypsinogen | Trypsin |
| Peptidases | Small intestine[a] | Oligopeptides | Amino acids |
| Nucleotidases | Small intestine[a] | Nucleotides | Nucleosidases, phosphoric acid |
| Nucleosidases | Small intestine[a] | Nucleosides | Sugars, purines, pyrimidines |
| Diverse bacterial enzymes | Large intestine | Various | Vitamin K, Various |

[a] Intracellular

Mucous layer interactions can also interfere with absorption.[42] A thick layer of mucus is secreted from the inner surface of the stomach protecting it from its acidic contents. Thinner layers coat the small and large intestine lubricating bolus food transport and protecting the delicate microvilli. This mucous can block nanoparticles from penetrating the intestinal wall, or provide a high turnover reservoir for temporary attachment and release.

Monoglycerols, fatty acids, and glycerol from food diffuse through the epithelial membrane into the local cells where they are reassembled to triglycerides. These triglycerides are formed into small droplets of approximately 150μm in diameter called chylomicrons. The chylomicrons are then transported by the golgi complex to the basolateral side of the cell and are exocytosed. The chylomicrons enter the central lacteal flow and are carried from the lymphatic system to vena cava to be injected back into the systemic circulation. This route through the lymphatic system can potentially be exploited for lipid based nanoparticle oral absorption.[43]

A formidable challenge to oral delivery is the first pass metabolism effect in the liver. The intestinal blood flow drains into the hepatic portal vein and then enters the liver. Here a host of metabolic enzymes and phagocytic cells reside that can sequester nanopreparations and their cargoes. Intact nanopreparations are generally limited to <5% entering systemic circulation when orally delivered.[44] Variables operating on the physiological environment such as state of fasting, disease and enzymatic content of the intestines can affect the oral absorption of these systems. Nevertheless, nanopreparations can potentially be conferred with

characteristics that increase gastrointestinal regional residence time, enable transepithelial transport, and optimize pharmacokinetic parameters.[45]

## 2.3 Tight junctions

Epithelial cells along the surface of the intestine are joined by intercellular tight junctions. These junctions between cells are formed by a variety of proteins including claudins and occludins. Unless transported by another transcellular mechanism, these proteins form a 'belt' around the epithelial cell, sealing off the majority of hydrophilic molecules from passage into the blood stream.[46] Nanopreparations of ~1–3nm[47,48] or smaller may penetrate through tight junctions without modifying intestinal physiology. Many nanopreparations with a larger diameter open tight junctions, facilitating their transport.[20,49] Endocytic mechanisms, such as clathrin and caveolin mediated pathways contribute significantly to nanoparticle uptake.[50,51]

Tight junctions have a range of pore sizes in different intestinal regions.[52] The molecular regulation of these junctions is also becoming better understood. It has been postulated that these junctions can open and close the intracellular space in a time dependent manner.[53,54] While the tight junctional areas only account for 0.1–0.01% of the surface area of the intestinal tract (approximately 200–2000cm$^2$), their potential impact on oral nanopreparations should not be overlooked.[11,48,55] Modern research places more emphasis on penetration enhancers that modulate tight junctions with reduced adverse effects.[52] The permeability of nanopreparations may be significantly increased by the use of

targeted protein penetration enhancers for tight junction proteins.

## 2.4 General principles of oral bioavailability

The general principles governing what is orally bioavailable is an area of continuous research.[56–61] Many factors affect amount of the compound that passes the intestinal epithelium and reach systemic circulation. These include partition coefficients (Log P, Log D), pKa, molecular weight/volume, aggregation, particle size and pH of the lumen and surface of the epithelium at the site of action. Physiological factors also play a role including state of anesthesia, blood flow, absorptive surface area, enzymes and membrane permeability. In short, to effectively evaluate an oral nanotherapy, it is important to use methods that have all the physiological and biochemical properties of human tissue, low variability between experiments, unbiased results, with comparable passive and active transport.[62] This challenge can be difficult based on the use of various animal and cell culture models for studying oral bioavailability of drugs, but these models form the basis for current comprehension of nano-bioavailability.

### 2.4.1 Mathematical evaluation of oral medicines

The extent of bioavailability is termed F for fraction of AUC compared to intravenous dose of comparable size

$$F=AUC_{PO}/AUC_{IV} \quad \text{(Equation 2.1)}$$

where $AUC_{IV}$ is equal to the plasma area under the curve profile for the IV administered drug and the $AUC_{PO}$ is equal to the plasma area under the curve profile for the orally administered drug. This fraction can vary between 0 and 1 and is generally <0.05 for orally delivered nanopreparations.[44]

In order for a compound to achieve high oral bioavailability it must have a high apparent permeability defined as:

$$P_{app}=(dQ/dt)/(A/C_0) \quad \text{..... (Equation 2.2)}$$

where A is the area of the permeable membrane ($cm^2$), $C_0$ is the initial concentration in the intestine and dQ/dt is the time rate of the appearance (or flux) of the drug from the donor side (typically the basolateral side of the intestinal epithelium). The $P_{app}$ equation comes from the equation for diffusion as notably established by Fick. Fick's first law ($J=-D\cdot\delta\varphi/\delta x$), where J is flux, D is the diffusion coefficient, $\delta\varphi$ is the concentration gradient and $\delta x$ is the spatial term, can be reduced to the equation for flux with constant temporal and spatial arrangement. $J=-P(c_2-c_1)$, where J is the membrane flux of the compound, $c_2$ is concentration on the basolateral membrane and $c_1$ is the apical concentration. When calculating the apparent permeability the $c_2$ term can be assumed to be 0, since we have limited concentrations on the basolateral side throughout the experiment. This results in the equation $J=P\cdot c_1$. Rearranging we achieve $J/c_1=P$ where P has units of cm/s, J has units of $mol/(cm^2\cdot sec)$ and $c_1$ can equal $mol/cm^3$. The J term is generally split into the time rate appearance of compound

on the basolateral surface (dQ/dt) in units of mol/sec, and the surface area (A) in units of $cm^2$ to give the final equation: $P=(dQ/dt)/(A \cdot c_1)$. This form of the equation is based on the assumption that the basolateral side and apical side of the solutions do not vary significantly from start of the experiment. In practice we keep the variation of the apical and basolateral side to <10% of the starting concentration. Typically a compound with good oral bioavailability has a flux over $10\times10^{-6}$ cm/sec through the intestinal tissue while a compound with medium bioavailability has a permeability between $1\text{-}10\times10^{-6}$ cm/sec and a compound with low bioavailability has a permeability $<1\times10^{-6}$ cm/sec.

### 2.4.2 Models of the intestinal tract

Four principal types of intestinal absorption models have gained prominence in the field of oral drug delivery: in vitro, ex vivo, in situ and in vivo. In vitro models such as parallel artificial membrane permeability assessment (PAMPA) and Caco-2 cells are commonly used for screening and ranking of drug permeability.[63] Ex vivo systems include isolated tissue models and everted intestinal sacs. In situ models include the rat intestinal isolation model, where a segment of the intestine is cannulated and filled with a probe solution. In vivo models range from animal gavage in rats to oral dosing to humans in clinical trials (Table 2.3).

The most prominent in vitro method used for both preliminary drug permeability and high throughput screening is the Caco-2 model. Derived from human colonic adenocarcinoma, the model exhibits polarized cell layers and

**Table 2.3.** Comparison of models of the intestinal barrier.[63,64]

| | **Caco-2 Cell Culture** | **Rat Isolated Tissue** | **Rat In Situ** | **Human Isolated Tissue** |
|---|---|---|---|---|
| Enzyme levels | - | + | + | + |
| Mucous layer | - | + | + | + |
| Morphology | - | + | + | + |
| Multiple cell types/Peyers Patches | - | + | + | + |
| Regional differences in intestinal transport | - | + | + | + |
| High throughput model | + | - | - | - |
| Correlation to human absorption | $R^2$=0.85 | $R^2$=0.95 | $R^2$=0.90 | $R^2$=0.85, 0.83 |

intercellular tight junction upon maturation. They also contain a host of CYP enzymes as well as human P-gp proteins.[63] Although some of the protein expression levels in Caco-2 cell lines are vastly different from those in human small intestine, the model provides sufficient data for ranking drug permeabilities.[64–66]

Models that use isolated animal tissue are referred to as ex vivo because they contain living tissue in a 'organotypic' environment with all resident cell populations (enterocytes, calciform cells, lymphocytes, etc.) as well as mucus layers and intestinal morphology.[63] The Ussing chamber set up is an ex vivo model which involves mounting intestinal tissue between two half cells for permeability testing (Figure 2.1). Sample volumes are loaded on the apical and basolateral side of the tissue and aliquots are taken at time intervals thereafter.[67] The tissue is oxygenated to avoid decreased tissue viability and to increase sample homogeneity through mixing. The tissue viability using this method ranges from 3-4 h depending on the region of interest.[68] Cell culture, animal, and human tissue has been used in Ussing studies.[69,70] The tissue can be tested via AgCl electrodes for epithelial resistance to $Cl^-$ ion transport. Large libraries of pharmaceuticals have been tested in Ussing chambers using human isolated tissue and found a strong correlation to exist between human fraction absorbed and isolated tissue permeability.[71] This model can perform multiple studies using a single animal in an effort to reduce and refine animal experiments.

The everted gut sac method involves removing a 2-3 cm length of intestine from an animal and inverting it over a glass rod so that the luminal side

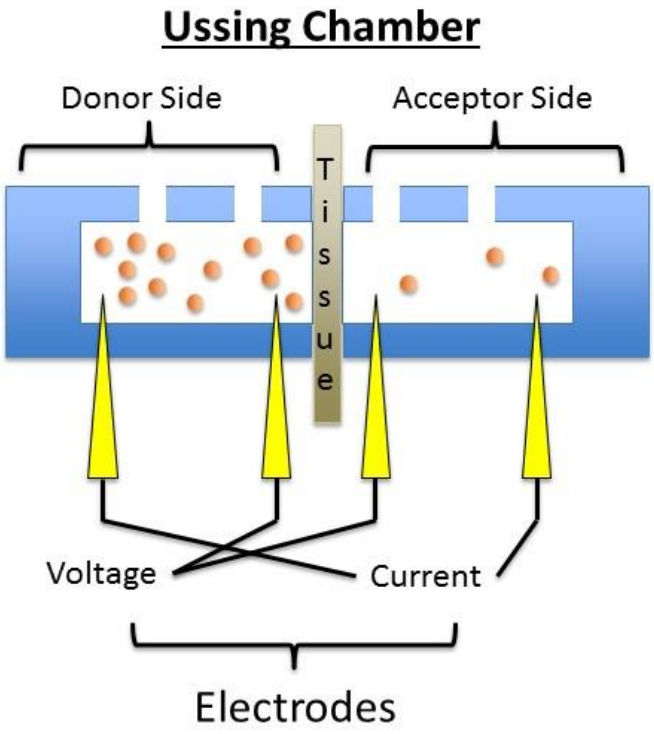


**Figure 2.1.** Schematic of an Ussing chamber set up containing isolated tissue.

faces outward. The tissue is subsequently filled with oxygenated buffer (for prolonged tissue viability) and tied off at both ends. This model has the disadvantage of permeability results being normalized to total protein content of the tissue (not exposed surface area as are Ussing chambers) and the variation inherent in protein content analysis.[63]

The in situ model involves permeability results performed in anesthetized animals through a laparotomy, and gently pulling the desired portion of the intestinal tract out of the abdomen for cannulation. Once the dose is injected the desired region of the intestine can be tied off at one or both ends of the tract for the duration of the study. This method has the advantage of controlled concentrations in the intestinal lumen, but is limited in duration and throughput.

The most frequently studied model in vivo is the rat since it maintains higher fidelity to human paracellular permeability and metabolism characteristics than dog.[72] Rats do have restrictions in dose volume and tend to overestimate (provide false positive) results.[73] Rats have a higher gastrointestinal pH than humans. The pH of chyme in the stomach of humans can be as low as 1-2 while rats exhibit pH levels as low as 3-5.[72] The microbial contents of the rat and human are different with different populations at different regions of the intestinal tract. The human stomach and upper small intestine are basically absent of microbial life, while rats have large population in the stomach and upper small intestine (due to the high pH of the stomach). The rat intestine includes a cecum which is much reduced in humans. Additionally The overestimation of nanoparticle transport by isolated rat jejunum may be due to the wide difference

in number of follicles per $cm^2$ of intestinal tissue (0.03 follicles/$cm^2$ versus 2.1 follicles/$cm^2$ in human and rat small intestine, respectively).[74,75] In general rat intestinal tissue is more permeable to macromolecules that human intestinal tissue as shown in this body of work and in others.[76]

The ability to successfully deliver cargoes in humans is not easy to extrapolate from small animal work and in vitro studies, which is the rationale to perform research in human isolated tissue models. Much of this work focuses on isolated intestinal permeability mounted in Ussing chambers. Isolated tissue intestinal models have more physiological characteristics of the human intestine than Caco-2 cell cultures.[77] Morphology, enzyme levels, mucous layers and cellular function and heterogeneous cell type are more accurately represented in isolated tissue models than in Caco-2 cell monolayers.[30] Also, the throughput of isolated tissue models is higher than in vivo work. This provides rationale for the use of isolated tissues to further study the potential of PAMAM dendrimers for oral drug delivery.

### 2.4.3 Correlation with human fraction absorbed

It can be argued that the most important aspect of selecting an intestinal model to study is the correlation with human absorption. The purpose of intestinal absorption models is to ultimately extrapolate those results to man in order to assess clinical absorption of the compound. Many correlations have been established over the years between in vitro, ex vivo and in vivo results to better predict human absorption.[71,77–82] Apparent correlations between Caco-2, isolated

rat tissue, in situ rat tissue and human isolated tissue experiments have been established. (Table 2.3) Caco-2 correlations exist, but have a very narrow region between 0 and 100% absorption prediction, making results highly variable. Caco-2 interlab variability appears to be very high and therefore standardization is difficult. Isolated rat intestinal tissue has a much larger prediction band between 0 and 100% absorption and has been found to have a better fit ($R^2$ of 0.95 vs $R^2$ of 0.90 for rat and Caco-2, respectively).

One of the largest correlation studies performed is the correlation between isolated human intestinal tissue and human fraction absorbed.[71] In this study a correlation was established between isolated human tissue permeability and human fraction absorbed was produced with combined results from three separate labs and 159 human donors and >60 drugs. The established correlation curve for human fraction absorbed had an $R^2$ of 0.85, and a p-value of <0.01. This study is a helpful addition in the prediction of human fraction absorbed in that it did not seek to exclude drugs based on low or high permeability classification, but included a broad range of pharmaceuticals with various transporter functions. Two of the pharmaceuticals excluded from the correlation were actively effluxed from the intestine by p-glycoproteins. Since this study used trace levels of the compounds for permeability assessment, these compounds showed erroneously low permeability (the efflux proteins are saturated at higher concentrations resulting in a high human absorption at clinical doses). Thus human isolated tissue has been established as an effective way to predict human transport and fraction absorbed for small molecular weight pharmaceuticals at

clinically relevant doses.

For macromolecular oral drug delivery systems the correlation with human isolated tissue still generally holds true, with compounds such as poly(ethylene glycol), ovalbumin, and dextrans and fitting into the correlation curve exactly as predicted.[76,83,84] Limitations of the model may come from compounds that are actively endocytosed or effluxed, such as was mentioned previously. Interactions with tissue mucous, and other epithelial layers may modify transport further. These changes may or may not be reflected in the permeability through the tissue and the prediction of fraction absorbed. Thus prediction of fraction absorbed for nanoparticles and polymers may be limited based on the correlation established in human isolated tissue, but is not entirely inaccurate.

### 2.4.4 Penetration enhancers

Most polymers and nanoparticles have low oral absorption necessitating the use of penetration enhancers to achieve therapeutically relevant levels. Penetration enhancers have been studied to increase the oral bioavailability of nanotherapies, especially proteins.[85] Generally penetration enhancers work by two methods: either chemical alteration of the macromolecule itself or formulations to exploit physiological or nonphysiological transport mechanisms.[86] Some of the major classes of penetration enhancers include chitosans, N-acylated amino acids, surfactants, steroidal detergents, acylcarnitines and protein penetration enhancers (Table 2.4).

PAMAM dendrimers have also shown to have penetration enhancing

**Table 2.4** Common penetration enhancers[84,85]

| *Type* | *Example* | *Advantage* | *Disadvantage* |
|---|---|---|---|
| chitosans | trimethylchitosan | very low toxicity | pH dependent effects on absorption |
| N-acylated amino acids | N-[8-(2-hydroxybenzoyl)amino]caprylate | very low toxicity | lacked clinical efficacy |
| surfactants | C10 fatty acids | biodegradable | limited enhancement, clinically used |
| steroidal detergents | chenodeoxycholate | reversible enhancement | limited enhancement |
| protein penetration enhancers | ZOT, melitten, arginine rich sequences | does not modify intestinal morphology | can be immunogenic |

effects, but those are limited to approximately a twofold enhancement ratio for hydrophobic molecules.[87] Additionally higher concentrations of PAMAM dendrimers have negative impact on epithelial morphology thus limiting their dose.[88] Protein penetration enhancers on the other hand have shown no damage to epithelial barriers at higher doses. [89,90] A combination of protein penetration enhancers with dendrimers may avoid further damage to epithelial barriers while still enhancing their permeability. Thus a combination of PAMAM dendrimer formulated with protein penetration enhancers may be optimal for polymeric oral drug delivery.

## 2.5 Types of nanopreparations

The general principles governing the physiochemical properties of drug molecules that influence oral absorption is an active area of research.[56–61] For nanopreparations, many factors can influence their absorption into the systemic circulation. These include particle composition, pKa of the material components, molecular weight, particle aggregation, particle size, pH, inherent stability of the particle, and surface charge.[11] Physiological parameters can also vary based on state of anesthesia, fasting or disease. These physiological parameters modify the permeability of the membrane, surface area of the epithelium and mucous layer properties.[39] These barriers have led to experimentation with a wide variety of nanoparticles for drug delivery (Table 2.5).

The single most formidable barrier for nanopreparations to enter the systemic circulation is the permeability of the intestinal membrane. Major types

**Table 2.5.** General overview of nanopreparations for oral delivery[137]

| | ***Physiochemical characteristics*** | ***Cargo*** | ***Therapeutic applications*** |
|---|---|---|---|
| Micelles | 20-50 nm<br>Dynamic structure<br>Hydrophilic corona<br>Hydrophobic core | Encapsulated in hydrophobic core<br>Mainly poorly soluble drugs | Solubilization<br>Inhibition of Pgp efflux |
| PCA | 40-200 nm<br>Static structure<br>Nondegradable backbone | Entrapped in core<br>Hydrophilic or hydrophobic<br>Small or large MW | Protection of drugs<br>Localized release |
| Chitosan | >100 nm<br>Static structure<br>Variable surface properties | Entrapped in core<br>Hydrophilic or hydrophobic<br>Small or large MW | Protection of drugs<br>Localized release<br>Gene Delivery |
| Dendrimers | 1-10 nm<br>Static structure<br>Variable surface properties | Surface attachment/entrapped space<br>Hydrophilic or hydrophobic<br>Small or large MW | Solubilization<br>Localized release<br>Protection of drugs |
| Liposomes | >50 nm<br>Dynamic structure | Entrapped in core or hydrophobic bilayer<br>Hydrophilic or hydrophobic<br>Small or large MW | Solubilization<br>Localized release<br>Protection of drugs |
| Solid Lipid Nanoparticles | >100 nm<br>Static structure<br>Hydrophobic/crystalline interior | Entrapped in core<br>Hydrophilic or hydrophobic | Solubilization<br>Localized release |
| PLA/PGA | >50 nm<br>Static structure<br>Customizable degradation rate | Entrapped in core<br>Hydrophilic<br>Small or large MW | Localized release<br>Protection of drugs |
| Nanosized Formulations | >100 nm<br>Static structure | Small MW<br>Hydrophobic | Increased solubility |

penetration enhancers include surfactants, chelating agents, bile salts, cationic and anionic polymers, acylcarnitines, and fatty acids.[11] Generally, penetration enhancers work by physiological alteration of the epithelium to exploit transport mechanisms.[91] Exploited transport mechanisms can be subgrouped into methods that disrupt the structural integrity of the epithelial layer, decrease the mucous viscosity, open tight junctions or increase membrane fluidity.[11] Penetration enhancement principles assume a sufficient quantity of therapeutic agent and penetration enhancers at the site of absorption.[92] This formulation goal can be difficult to accomplish due to variance in intestinal motility, gastric emptying and dilution factors in the gastrointestinal tract. Approximately 9.0L of fluid enters the small intestine each day, of which only about 2.0L is food and liquid ingested, giving a dilution factor > 4 fold.[39] Nanoparticles can increase effectiveness of penetration enhancers by carrying them to the intestinal barrier and releasing them in a site specific manner to aid in particle absorption. Penetration enhancers, which have been reformulated into nanopreparations, aid bioavailability by increasing the local delivery of drug and enhancer. Makhlof et al. showed that a solution of co-delivered penetration enhancer spermine and nanoparticles was not as effective as nanoparticles encapsulating spermine in delivery of salmon calcitonin to rats.[93] This provides rationale for encapsulating penetration enhancers such as chitosan and glycerides into nanoparticles alongside the therapeutic cargoes.[49,94–98]

### 2.5.1 Chitosan nanoparticles

Chitosan has been widely researched for uses as both a nanopreparation and as a permeation enhancer in oral delivery.[99–101] Chitosan is made from deacetylated chitin. Chitosan is a linear polysaccharide made of varying ratios of N-acetyl-D-glucosamine and D-glucosamine groups. It has an extremely high oral $LD_{50}$ in mice of 16 g/kg orally (as nontoxic as sugar and salt).[102] Primary amines and hydroxyl groups on chitosan can be modified to attach functional moieties. Chitosan has a positive charge at pH <6.5 due to the fact that it has free amino groups with a pKa of 6.1. Native chitosan has limited solubility at pH >7.[103] Lack of charge and solubility of chitosan at neutral and basic pH levels cause it to be less effective as a penetration enhancer in the small intestine where pH can rise above 6.5. Modifications were thus developed to increase chitosan's solubility in the small intestine. Thiolated chitosan, quaternization of the primary amine with methyl groups (trimethylchitosan), PEGylated chitosan, N-succinyl chitosan and carboxymethyl chitosan derivatives have all been evaluated for oral delivery.[104–107] Some of these have been used in oral nanopreparations with varying effects.

Chitosan nanoparticles have shown to cause a decrease in TEER (transepithelial electrical resistance) and increase in FITC-dextran permeability in Caco-2 models.[108,109] The mechanism by which they open tight junctions appears to be through F-actin and ZO-1 protein depolymerization.[110] The penetration enhancing effect of chitosan solutions appears to be dependent on the molecular weight and degree of deacetylation of the chitosan. The best results for

absorption enhancement have been observed for chitosans having 65% deacetylation and a >170 kDa molecular weight in rat small intestine. This information can be useful for the preparation of nanoparticles as well.[111] Chitosan nanoparticles have exhibited particular advantages over free chitosan solutions in that they are endo- and transcytosed and better protect cargo drugs.[112]

Substantial research has been conducted on the oral delivery of insulin using chitosan as a nano-carrier.[101,113–115] Many of these have utilized the mild ionic gelation techniques that can maintain insulin's three-dimensional structure. In one example, nanopreparations were synthesized by ionotropic gelation in the size range of 250–400 nm using 88.9% deacetylated chitosan.[96] When dosed to diabetic rats, hypoglycemic blood glucose levels were maintained for >15 h. The relative oral bioavailability of the insulin was as high as 14.9%.[96]

In order to increase the bioavailability of insulin, modified chitosan has been studied. The basis for modification is to maintain the charged nature of the amine groups in order to increase the penetration enhancing effects at neutral and basic pH. For this purpose quaternized chitosan nanoparticles have been developed.[116] Trimethyl- and dimethyl chitosan showed enhanced insulin delivery in rats over chitosan solutions and unmodified chitosan nanoparticles during colonic delivery.[116] These were estimated to improve bioavailability based on the increased solubility at neutral and basic pH. The nanoparticle delivery showed the highest effect when free chitosan solutions and chitosan nanoparticles were admixed prior to delivery.

Active targeting of chitosan using peptides has also been investigated for

goblet cell delivery. Trimethylchitosan nanoparticles with goblet cell targeting peptides (CSK) showed improvement over trimethylchitosan insulin delivery.[117] In Caco-2/HT29-MTX co-cultures, transepithelial transport was increased using CSK peptide-conjugated nanoparticles. Similar nanoparticles loaded with insulin showed increased relative bioavailability compared to subcutaneous injection in rats for targeted versus nontargeted nanoparticles (5.66% vs. 3.69%, respectively).[117] This study provided the rationale for active targeting of chitosan nanopreparations in oral delivery.

Combinations of nanopreparations have been studied for improved oral delivery of therapeutic cargoes with chitosan. An example is alginate: a biodegradable anionic polymer composed of β-D-mannuronic acid and α-L-glucuronic acid. The anionic properties of alginate make it ideal for complexation with chitosan as this increases their stability. Combinations of chitosan and alginate also aid in oral insulin delivery by facilitating the complexation of the protein and nanoparticle.[100] Nanoparticles formulated by ionotropic pregelation of an alginate core followed by chitosan complexation with the surface were ~750 nm in diameter and obtained an insulin loading capacity of 9.9%.[49] When dosed orally to diabetic rats they showed a significant hypoglycemic effect from 8–14 h with decreased levels lasting for 18 h. The duration of hypoglycemia was longer than in previous studies with chitosan nanoparticles.[96,113] Insulin solution mixed with the nanoparticles showed only a minor decrease in blood glucose levels, highlighting the importance of encapsulation in the nanoparticles.[49] The relative bioavailability of insulin in this nanoparticle formulation was 7% versus

subcutaneous insulin.[49]

Some of the most promising results in vivo involved the encapsulation of insulin in chitosan, facilitated by the incorporation of glutamic acid residues and zinc (a known complexing agent for insulin[118]). Chitosan-γ-glutamic acid nanoparticles of diameter ~197 nm showed a loading efficiency of insulin of 57%. They reduced blood sugar levels by 60% for >10 h in streptozotocin induced diabetic rat models.[20] The benefits of these chitosan-glutamate nanopreparations were improved solubility at a range of pH values. Unfortunately these systems degraded in vivo in the neutral-basic pH of the intestine causing premature release and degradation of the protein.[20] In order to overcome this problem Sonaje et al. lyophilized the nanoparticles and inserted them into enteric capsules. This approach increased the oral bioavailability of insulin to ~20%. However conclusive evidence in large animals and man is necessary.[119–121]

Inhibition of intestinal proteolytic enzymes has been explored for enhancing oral delivery of proteins.[122] Chitosan alone is insufficient to inhibit trypsin and carboxypeptidases.[123] Further development of chitosan antienzyme technology led to the conjugation of ethylenediaminetetraacetic acid (EDTA) to chitosan, but these conjugates also were not effective against trypsin, chymotrypsin and elastase.[122] Competitive enzyme inhibitors (i.e., papain, chymostatin) were used in conjunction with EDTA-chitosan to slow degradation of encapsulated insulin. 40–60% of insulin remained after 4.5 h when including inhibitors compared to 90% degradation without.[124] This combination highlights the potential role of enzyme inhibitors in oral nano drug delivery.

Chitosan-DNA complexes have been studied for the purpose of improving transfection efficiency via the oral route. Attempts have been made to optimize transfection efficiency based on charge ratio, chitosan molecular weight, endosomes escaping peptide conjugation and DNA concentration.[125] These studies have resulted in formation of stable DNA-chitosan nanoparticles of approximately 100–500 nm in size (102kDa chitosan). However they produced expression levels 200 fold less than Lipofectamine on Cos-1 cells. Surprisingly, when instilled in an upper rabbit small intestine, chitosan showed better transfection than lipid based DNA carriers.

Chitosan nanoparticles have also been studied for oral anti-hypersensitivity gene therapy. In one of these systems, cDNA for peanut antigen was encapsulated in monolithic high molecular weight chitosan (390 kDa) nanoparticles. The particle size was assessed by TEM and appeared on the order of 150–300 nm in size. The testing of anaphylactic shock scoring after oral administration of chitosan-DNA complexes, carrying the peanut antigen, showed that this nanopreparation was able to reduce hypersensitivity response to peanut antigen in vivo.[126] Lack of clinical translation of these systems may be related to low transfection efficiency due to mucous entrapment of the chitosan nanoparticles occurring in the intestine. Uptake of the chitosan nanoparticles and subsequent release of the DNA may give higher transfection rates than pre-endocytosis release. The mucous adhesion of the chitosan may increase local concentrations of the target gene at the epithelial surface, but limit uptake of the nanoparticles themselves, thus limiting transfection.[127] Improved transfection

efficiency will probably be needed to merit further development and translation.

In spite of significant efforts for developing oral chitosan based delivery systems they have not yet reached clinical trials.[100,127] A major concern may be the toxicity of chitosan. In vitro blood coagulation studies have shown that 2% solution of unmodified chitosan (126kDa) causes a 40% decrease in coagulation time with heparinized blood, whole blood, and defibrinated blood.[128] Varying degrees of quaternization have showed modulated in vitro toxicity, bringing to light the importance of evaluation of novel chitosan derivatives. This effect is illustrated by the study in MCF-7 cells where increasing charge density (quaternization) exhibited a decreasing $IC_{50}$ at an exposure time of 6 h.[129] Future clinical development may require extensive toxicity evaluation of chitosan which could be a possible reason for lack of translation.[130] Also the ability of chitosan to increase oral bioavailability may not be sufficient. Difficulties with scale up of production may be another source of concern. Despite these problems, the ease of synthesis and penetration enhancing effects of chitosan have prompted continued research of the material. Novel modifications, such as thiol-protected chitosan, hold promise for future improvement.

### 2.5.2 Poly(alkyl cyanoacrylates) (PCA)

Poly (alkyl cyanoacrylates) have been extensively used for drug delivery.[131,132] These polymeric systems are made from the common acrylate monomer system with an additional cyanate group. The use of PCA in drug delivery is based on their ability to encapsulate small hydrophobic drugs and also

proteins.[133] PCA was the first polymer nanoparticle used for oral delivery of peptides.[133] This landmark study using insulin set the stage for many other nanoparticle-peptide oral drug delivery studies.[134] PCA has also been shown to improve the oral bioavailability of small molecular weight drugs.[135,136] A large number of studies have been done on the delivery of proteins and drugs using PCA demonstrating its versatility as a drug delivery agent.[137,138] PCA is considered bioerodable due to the hydrolysis of its side chain ester moieties producing the corresponding alkyl alcohol and poly(cyanoacrylic acid).[139–141] In the gut, pancreatic esterases are able to degrade PCA nanoparticles and release their cargoes. This hydrolysis proceeds at variable time rates depending on the side alkyl chain length. PCA above molecular weight 10,000 Da is probably not excreted from the body.[142]

PCA nanoparticles can be synthesized in acidic water solutions with strong mixing and added surfactants for stabilization.[137] Polymerization begins immediately upon the addition of the monomer to any solution containing nucleophilic groups (i.e., OH). The rate of reaction can be slowed by lowering the pH of aqueous polymerization solutions. The size of the nanoparticles can be adjusted between 40 and 200 nm based on the concentration of surfactants used in pure PCA nanoparticle synthesis. The combination of different polymers (chitosan, dextran) can change the size of the end product as well as the zeta potential. For in vivo work all products need to be maintained sterile during polymerization. Heat, gas, filtration or radiative sterilizations are not appropriate for PCA nanoparticles.[137] Nanocapsules (nanoparticles with hollow cores) can be

formed using PCA. These can be either water or oil-cored to carry cargoes. Isobutylcyanoacrylate (IBCA) nanocapsules have been used for oral delivery of insulin in streptozotocin diabetic rats (see Figure 2.2 for structural differences of PCA monomers). Results obtained showed hypoglycemia of 65% lasting for up to 18 days after administration. Delivery to the ileum showed the greatest hypoglycemic effect using the IBCA nanoparticles.[143] This demonstrates the ability of nanoparticles of IBCA to deliver proteins orally, but other results have shown lack of efficacy. Nanoparticles of IBCA entrapping calcitonin underwent slow degradation of the peptide in simulated intestinal fluids, but in vivo absorption was not significantly increased.[144] Initial studies by Lowe et al. showed that rapid release of calcitonin occurred and little protection from intestinal proteins was afforded by IBCA nanoparticles.[144]

Nanoparticles using PCA have also increased the oral bioavailability of small molecular weight drugs. Hexylcyanoacrylate (HCA) nanoparticles have shown to increase the oral bioavailability of vincamine in rabbits.[136] The oral bioavailability of vincamine in HCA nanoparticles was increased from 25% to 40% when the drug was entrapped. Also the oral bioavailability of mitoxantrone was increased by incorporation in poly(butylcyanoacrylate) nanoparticles. This result could be explained by the local release of the drug next to the epithelial barrier following the mucoadhesion.

Poly(isohexyl cyanoacrylate) (PIHCA) nanoparticle formulations have been investigated for the delivery of cyclosporine A. Nanoparticle delivery could potentially decrease acute kidney damage caused by this drug in humans. An

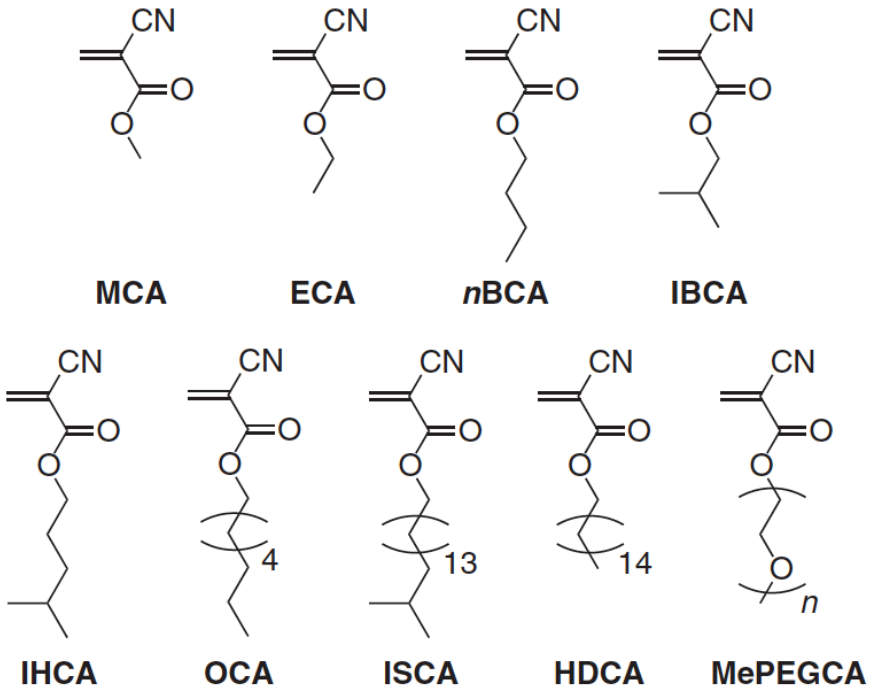


**Figure 2.2.** Chemical structures of common PCA derivatives. Methyl cyanoacrylate (MCA), ethyl cyanoacrylate (ECA), n-butyl cyanoacrylate (nBCA), isobutyl cyanoacrylate (IBCA), isohexyl cyanoacrylate (IHCA), octyl cyanoacrylate (OCA), isostearyl cyanoacrylate (ISCA), hexadecyl cyanoacrylate (HDCA), and methoxypoly(ethylene glycol) cyanoacrylate (MePEGCA).[184]

in vivo study of PIHCA nanoparticles in mice showed a 13 fold increase in bioavailability of the nanoparticle over an emulsion of cyclosporine A.[145] The study also showed a greatly reduced liver AUC as well as reduced kidney exposure to the drug. This study demonstrates the further ability of these systems to avoid reticuloendothelial uptake.

Poly(butyl cyanoacrylate) (BCA) loaded with calcitonin showed a 45% decrease in calcium levels compared to control intravenous injection following oral administration to rats. Absorption enhancers such as deoxycholic acid and sodium lauryl sulfate were also used to enhance permeability.[146]

Specific properties of PCA nanoparticles can be modified in order to modulate interactions with mucous proteins. This can optimize absorption enhancing properties of nanoparticles and cargo release. Size and surface characteristics are the major properties that affect mucous interactions.[147–149] Other properties, including surface modification with other polymers such as large MW PEG and chitosan, can increase mucoadhesion.[150]

Toxicity of PCA has been noted for shorter chain cyanoacrylates (methyl cyanoacrylate) in humans when used as a wound closure agent, and subsequently was replaced by longer chain cyanoacrylate adhesives.[132] It has been reported that the degradation products of PCA could potentially be converted to formaldehyde through the inverse Knoevenagel reaction. This led to several reports attributing toxicity of nanoparticles to this mechanism.[151–153] It has been postulated that this mechanism is too slow to compete with much more rapid metabolic pathways that degrade PCA.[132,152,154] The results of an

intravenous phase III clinical trial of PIHCA nanoparticles for the purpose of doxorubicin delivery are of relevance (Livatag®, BioaAlliance Pharma, France). This trial was later suspended based on severe pulmonary adverse events.[134] This could have been due to aggregation of the nanoparticles, but the exact mechanism is unknown. Toxicity of these systems needs to be further addressed for intravenous and oral dosing.[134]

### 2.5.3 Dendrimers

Dendrimers were first described in 1978 by Fritz Vögtle and his group at University of Bonn.[155] Since then a variety of these branched structures have been developed.[156] Dendrimers are characterized by their hyperbranched structure resulting from repeating branches off of a central core atom. This branching results in a tree like structure, that forms a spherical or near spherical macromolecule with multiple sites for attachment at the surface (Figure 2.3).[157] Also the internal spaces remaining between the branched structure create loci for hydrophobic encapsulation of other drugs or nanoparticles in the core.[158] This multifunctional surface and encapsulating core, combined with low polydispersity and prolonged plasma half-life make these polymers suited for drug delivery.[6]

#### 2.5.3.1 Poly(amido amine) (PAMAM) dendrimers

PAMAM dendrimers have been evaluated for oral drug delivery.[157] These dendrimers are synthesized through repeated Michael addition to the free terminal amine groups.[159] This synthesis results in alternating amine terminated

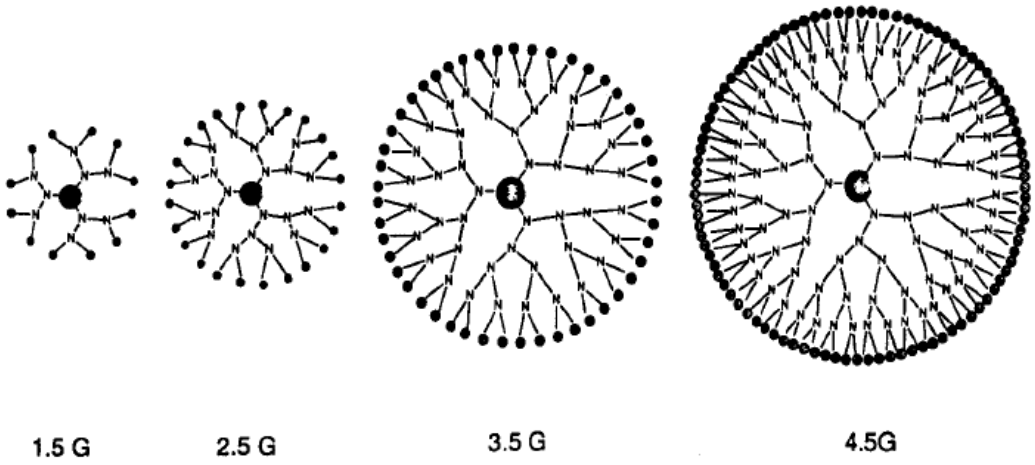


**Figure 2.3.** Branching PAMAM dendrimers enable attachment of moieties to multiple surface groups or the encapsulation of molecules in the interior spaces. Reprinted with permission from M. F. Ottaviani, S. Bossmann, N. J. Turro, and D. A. Tomalia, Characterization of starburst dendrimers by the electron paramagnetic resonance technique. 1. Copper complexes in water solution, Journal of the American Chemical Society. 116(2), 661Ð671 (Jan., 1994). Copyright 1994 American Chemical Society.

or carboxyl terminated surfaces. Full generation amine terminated (G1.0, G2.0, etc.) or half generation carboxyl terminated (G0.5, G1.5, etc.) dendrimers allow for modification of the surface charge and modulated toxicity profiles.[160] PAMAM dendrimers were first shown to cross the gut mucosa in an everted intestinal sac model.[161] Altering the termini can change the bioavailability of these dendrimer conjugates. These characteristics give favorable qualities for PAMAM dendrimer drug delivery.

The potential of PAMAM dendrimers to permeate across the intestinal epithelial barrier was shown by Wiwattanapatapee et al.[161] Using everted rat gut sacs it was observed that serosal transfer rate of anionic dendrimers was high (2.3–2.7 microL/mg protein/h) for G3 and G4.[161] Transepithelial transport was observed to be dependent on a variety of factors including size, surface charge, concentration and incubation time.[88]

PAMAM dendrimers have shown toxicity both in vitro[157] and in vivo.[160] Caco-2 cell culture studies and CD-1 mice experiments showed that toxicity was generation, size, surface charge and incubation time dependent.[160,162] Toxicity for PAMAM dendrimer surface groups ranks in the order of hydroxyl-terminated<carboxyl-terminated<amine-terminated systems.[163] Carboxyl terminated, G3.5 and G4.5 PAMAM dendrimers were cytotoxic using an LDH release assay from Caco-2 cells at concentrations higher than 10.0 mM whereas amine terminated dendrimers, G3.0 and G4.0, were cytotoxic at much lower concentrations (1.0mM). Concentrations of 0.01mM G4.0 were found to be non-cytotoxic by LDH assay and visual inspection in Caco-2 cells.[164]

Orally dosed PAMAM dendrimers showed similar toxicity to intravenous injections of the same in CD-1 mice. At toxic doses, disseminated intravascular coagulation-like effect and fibrin degradation products with resultant intravascular coagulation and hemorrhage were observed. It was noted that the amine terminated dendrimers were more toxic than carboxyl terminated in CD-1 mice.[160] PAMAM dendrimer surface groups have been modified to reduce toxicity. PAMAM dendrimers with lauroyl chains attached to the surface have shown decreased toxicity and increased permeability across Caco-2 cell monolayers.[165] Other surface modifications such as PEGylation, acetylation and FITC labeling have been observed to reduce toxicity and improve transport across Caco-2 monolayers.[166–168]

Anionic dendrimers have been observed to open tight junctions and increase mannitol transport across Caco-2 cell monolayers. In the size range studied, larger dendrimers appeared to have a greater effect. Higher permeability of mannitol across Caco-2 cell monolayers was noted for G2.5, G3.5, and G4.5 dendrimers, compared to smaller G-0.5, G0.5 and G1.5 dendrimers.[169]

Fluorescently labeled G2 dendrimers showed increased permeation with increasing times ranging from 90 to 150 min in Caco-2 cell culture. It was also shown that the transport was energy-dependent with decreased permeability at 4°C compared to 37°C.[162] A portion of transport may be dependent on endocytic mechanisms. PAMAM dendrimers have been shown to be transported across Caco-2 cell monolayers via paracellular and transcellular routes.[50,51,162,164,167]

The ability of PAMAM dendrimers to permeate the epithelial barrier and

enter systemic circulation was recently investigated.[170] It was shown that 106,000 Da G6.5 dendrimers were able to be absorbed orally in CD-1 mice. One mg/kg of radio-labeled G6.5 dendrimers were dosed by oral gavage to mice, and animals were sacrificed at 4 h. 9.4% of the macromolecular dose was found to be absorbed into the plasma after exclusion of unbound radio-label by size exclusion chromatography.[170] This in vivo study shows promise for enhancement of bioavailability of highly potent drugs using PAMAM dendrimers.

The potential of PAMAM dendrimers to increase transepithelial flux has been harnessed for the delivery of propanolol.[165] Lauroyl modified dendrimers with 2–6 moles of lauroyl per dendrimer were attached to G3.0. The study showed increased transport of propanolol when lauroyl chains were attached compared to dendrimer and propanolol alone. The endocytosis inhibitor cyclosporin A did not decrease transport, but reduced temperature (4°C) did. This phenomenon may be based on the energy dependent endocytosis of PAMAM dendrimers. More studies are needed to evaluate the stability of dendrimer-lauroyl chain conjugates.

PAMAM dendrimers have also been shown to increase the transepithelial transport of naproxen when covalently conjugated.[171] The stability of the conjugated system to G0 dendrimers was dependent on the bond type with the amide bond being more stable than the ester. Using liver homogenate and plasma it was found that the amide conjugate was stable for up to 48 h. Further attachment of lauroyl chains increased permeability across Caco-2 cells.[172]

PAMAM dendrimers have shown increased transepithelial transport of

anticancer compounds. Kolhatkar et al. complexed SN38 (7-Ethyl-10-hydroxy-camptothecin) with PAMAM G4.0. SN-38 is a highly potent topoisomerase poison which has poor water solubility and dose limiting toxicity. The prodrug of SN38, CPT-11, is used in the clinic, but is 1000 fold less active than SN-38. Complexation or conjugations with dendrimers can potentially reduce toxicity and improve oral bioavailability. Complexation with dendrimers led to a 100 fold increase in transepithelial transport across Caco-2 cell monolayers. However the complex was not stable.[173] Attempts to remedy this problem by covalent attachment were made.[174] Stability of the conjugates of SN38 with carboxyl-terminated G3.5 using glycine and β-alanine linkers demonstrated that the β-alanine linkers are more stable. Transepithelial transport across Caco-2 cells was increased for both conjugates. Studies performed with these conjugates found reduced cytotoxicity compared to the free drug for both.[175] Further studies with PAMAM dendrimers for delivery of small molecular weight drugs has been reviewed previously.[157,163]

PAMAM dendrimers have major advantages over other penetration enhancers in terms of being able to covalently attached therapeutic drugs to the surface and increase their transport. Most other penetration enhancers only modify the epithelial border to increase transport. Dendrimers are a macromolecular drug delivery vehicle which add improved circulation time, passive targeting and solubility to covalently attached drugs.

Some of the problems with clinical translation of PAMAM dendrimers may be associated with the batch to batch variability in synthesis. Low and variable

drug loading have caused problems associated with drug attachment. While some of these problems may be associated with finding appropriate solvent systems for hydrophilic dendrimers and hydrophobic drugs, other problems may arise from the altered pKa and reactivity of surface amine and carboxylic acids on dendrimers.

PAMAM dendrimers may have an altered pKa and reactivity of surface amine and carboxylic acid groups. Surface groups have a tight spacing on dendrimers (approximately 0.78$nm^2$ per group). Surface amines have been shown to fill outer perimeter space exceeding the packing limit of amines and forcing some groups inward.[176,177] This may increase the pKa of surface amines forcing away excess protons and positive ions. Conversely, closely packed carboxylic acids on half generation dendrimers may have a lowered pKa due to the electron rich environment of many tightly packed COO- groups. This may influence conjugation chemistry and other techniques used to attach external groups. Specifically, the popular carbodiimide chemistry could be inhibited by the lack of protonated carboxylic acid groups on half generation dendrimers.[178]

PAMAM dendrimers have potential to improve the bioavailability of poorly absorbed drugs. Toxicity of these polymers must be monitored in order to avoid systemic effects and local irritation. In addition linker chemistries need to be developed that are stable in the GI tract, during transport across the blood barrier and in the blood stream and allow release at the target site. Another barrier to translation of PAMAM dendrimers is challenge with scale up of the synthesis, which takes several reaction steps to produce.[156]

#### 2.5.3.2 Poly lysine dendrimers

Poly lysine dendrimers have been synthesized for improved stability and reduced synthetic steps (reduced cost) over PAMAM dendrimers.[156] These dendrimers have also been studied for oral absorption. Florence et al. synthesized and characterized poly lysine dendrimers originating from glycine cores with lipid termini. Two and a half nm diameter dendrimers were chosen for dosing to rats at 14mg/kg and 28 mg/kg. A maximum of 3% of the tritiated dendrimer was found in the blood at 6 h.[179] This uptake was not in excess of that for 50 nm latex beads. More studies are needed to investigate in detail the stability of poly lysine radiolabel conjugates in the GI tract, during transport and in the systemic circulation.

### 2.5.4 Poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) nanoparticles

PLA and PGA have the unique advantages of being both degradable and resorbable. The two monomers can by copolymerized (poly(lactic-*co*-glycolic acid)) (PLGA) at varying feed ratios to customize the crystallinity and hydrolysis rate (Figure 2.4).[180] Both nano- and micro- drug delivery have been studied using PLGA particulate systems. The biodistribution of the PLGA particles can be influenced based on the size of the particles. Particles that are >250–300 nm are predominantly taken up by the spleen, while particles that are several microns in diameter are taken up by the lung.[181] PLGA nanoparticles have been shown to increase intestinal tissue uptake of certain drugs using in situ loop model

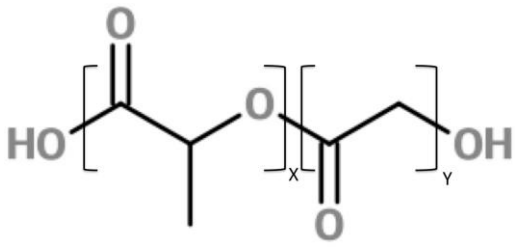


**Figure 2.4.** Structure of poly(lactic-co-glycolic acid) (x=lactic acid, y=glycolic acid).

systems validating their use for oral drug delivery.[38] Uptake into the intestinal tissue of the free nanoparticles was especially high for 100 nm PLGA particles in the ileum of anesthetized rats. It was noted that uptake was higher in Peyer's patches compared to villous tissue.

Increased absorption has also been observed using PLGA nanoparticles for oral delivery of drugs. PLGA nanoparticles were used for the purpose of administering antituberculosis drugs isoniazid, rifampicin and pyrazinamide orally.[182] These nanoparticles had a size of 186–290 nm and showed an increased mean residence time (in the blood-plasma compartment) and bioavailability in guinea pigs. The levels of drug in the plasma were sustained for 7–12 days when encapsulated, while the drug alone was only detectable for 24 h. Guinea pigs infected with *mycobacterium tuberculosis* showed no signs of infection following administration of 5 oral doses of antibacterial loaded PLGA nanoparticles, compared to 46 doses of the free drug. This study shows promise for reducing dose frequency for the antituberculosis drugs.[182] Improvements on this study were performed by attaching wheat germ agglutinin to the PLGA nanoparticles. Bioavailability and residence time were increased from 4–6 days to 6–7 days for rifampicin, and from 8–9 days to 13–14 days for pyrazinamide and isoniazid.[183]

PLA-pluronic-PLA triblock copolymers have been used for effective oral insulin delivery.[184] These nanopreparations were synthesized due to their vesicular nature and theoretical biocompatibility. The ability of these polymer combinations to encapsulate insulin was demonstrated. The release profile for 56

nm PLA-pluronic-PLA vesicles showed a biphasic pattern, with initial burst release and subsequent sustained release over a 4 h time period. Induction of hypoglycemia in rats was demonstrated by gavaging 50 IU/kg dose in vesicles which yielded a sustained effect for over >18.5 h.[185]

PLGA nanoparticles have been covalently linked to RGD peptides for the purpose of increasing M cell uptake.[186] These nanopreparations showed increased uptake in human M cell co-cultures. In vivo ovalbumin-loaded PLGA nanoparticles showed significant concentration in mice M cells and were able to elicit an immune response.

Immune cell targeting is one of the goals of PLGA nanoparticle oral delivery. Previous work on oral vaccination against virulent strains of *E. coli* has been carried out in humans using microspheres. This has led to vaccine efficacy of 30% in early studies.[187] Later, Katz et al. used PGA microspheres carrying CS6 antigen from enterotoxigenic *E. coli*, in order to induce immunity, but there was no apparent difference in response to the encapsulated antigen compared to the unencapsulated. While these were microparticles, their equivocal results may be the chief reason why PLGA particle systems have not been continued in further clinical trials for vaccine delivery. Other reasons why these systems appear to have been excluded from further clinical testing in oral delivery may be due to instability, lack of uptake from the intestine, instability of the antigen during synthesis and low antigen loading.[188,189]

### 2.5.5 Polymeric micelles

Polymeric micelles represent a colloidal dispersion ranging from 5 to 100 nm in diameter. They are generally composed of amphiphilic materials, which spontaneously self-assemble to form a hydrophobic core and hydrophilic corona in aqueous environments. This formation occurs when the concentration of amphiphilic material rises above the critical micelle concentration (CMC) and when the temperature of the system remains below the CMT (critical micelle temperature). The association of the amphiphilic molecules is based on the decrease of free energy of the system due to the loss of hydrophobic interactions with water molecules, and the reestablishment of the hydrogen bond network in water.[190] Micelles are different from liposomes in that they lack an aqueous core, but instead maintain a hydrophobic core where poorly soluble cargoes (drugs) can be encapsulated. In the intestinal tract bile salts are believed to naturally form micelles aiding in fat digestion and transport.[3,191,192] Micelles have been used in oral drug delivery and enhancement in $C_{max}$, $AUC_{0-24}$ and $t_{max}$ of poorly soluble drugs in vivo.[8,193] Polymeric micelles generally do not affect TEER.[194]

Pluronic (poloxamer) micelles have shown potential for oral drug delivery.[195] Pluronics are triblock copolymers made of a central hydrophobic chain of poly(propylene oxide) (PO) with two hydrophilic chains of poly(ethylene oxide) (PEO) on the outside. These can be tailored for specific hydrophilic / hydrophobic balance by customizing the length of the hydrophilic EO and hydrophobic PO portions of the polymer. Pluronics spontaneously form micelles above a critical micelle concentration in the range of 10–50nm.[196] Pluronic F127

of average compositions $EO_{99}PO_{67}EO_{99}$, showed increased oral delivery of genistein in rats compared to genistein powder.[197] The plasma AUC was 3 fold higher at a dose of 4 mg/kg genistein in anesthetized rats ($p > 0.5$). The micelle particle size was on average 27nm. Pluronics inhibit P-gp efflux showing potential to aid in transepithelial transport.[8]

The ability of micelles to improve bioavailability of small molecule drugs (BCS class II) were exhibited using monomethyletherpoly(oxyethylene $glycol_{750}$)-poly(caprolactone-co-trimethylene carbonate) ($mmePEG_{750}P$(CL-co-TMC)) micelles and risperidone.[198] Micelles, 20 nm in diameter (10% weight polymer solution), were dosed to rats exhibiting a 2 fold increase in risperidone bioavailability compared to risperidone solution. $T_{max}$ was extended by 3 h to match that of the radiolabeled micelle constituents. This correlation between drug and micelle concentration maxima suggests that the drug could remain encapsulated during transport. Drug half-life was extended 1.3 times showing a sustained release effect.

The increased transepithelial transport of micelle encapsulated drugs remains controversial.[194] Some evidence suggests that increasing concentration of micelles decreases transport of micellar components across the membrane. $^{14}C$ labeled $mmePEG_{750}P$(CL-co-TMC) micelles showed decreased permeability across Caco-2 cells with increasing concentration of polymer.[198] This could be due to passage of unimers and lack of passage of micelles, leading to saturated transport of unimers at or above the CMC. Opposing this report, poly(oxy ethylene cetylether hydroxyl propylcellulose) (HM-HPC) mixed with poloxomer 85

caused a 3 fold increase in permeability of cyclosporin A across Caco-2 cells.[199] It was hypothesized that increased solubility may be the primary reason for increased transport. In spite of opposing in vitro studies, transport of micellar components appears to be significant in vivo. Micellar components were observed to reach a bioavailability as high as 40% upon oral administration of mmePEG$_{750}$P(CL-co-TMC) micelles to rats. These results validate the oral absorption of micellar components in vivo.[198]

D-α-tocopheryl poly(ethylene glycol) succinate (TPGS), poloxomers and a variety of other common micelle components have shown inhibitory effects for P-glycoprotein (P-gp) efflux pumps.[194] This has provided the rationale for oral drug delivery using polymeric micelles. The mechanism by which TPGS inhibits Pgp efflux appears to be through allosteric modulation of the Pgp protein, while that of poloxomer appears to be through membrane fluidization and decreased ATPase activity.[200,201] Increased transepithelial transport due to Pgp efflux is most pronounced at concentrations below the CMC, which is likely due to higher presence of the unencapsulated drug in solution.[202,203] The exact mechanism of Pgp efflux inhibition by these micellar components is not fully understood.[194]

The effects on Pgp efflux may be observed in an oral administration study of paclitaxel in micelles of poly(ethylene glycol) poly(2-(4-vinylbenzyloxy)-N,N-diethylnicotinamide) (PEG-b-P(VBODENA)).[204] Upon intraduodenal administration in rats of the paclitaxel micelle formulation (drug loading of 37.4 wt%, 105–120 nm diameter), bioavailability was 12.4% compared to a previously reported oral bioavailability of Taxol® of 6.5%.[205] The ability of the micelle

formulation to increase the bioavailability may be dually related to the increase in solubility of paclitaxel and the inhibition of Pgp efflux.

For BCS class II drugs poor solubility is a significant concern that may be surmountable using micellar compositions. The physiochemical structure of micelles with the hydrophobic core and hydrophilic corona make them well suited for formulation of such drugs. Increases in solubility have been observed to be as high as 15,000 fold for paclitaxel.[204] The ease of synthesis of these systems is also well suited for manufacture, in many cases requiring only simple mixing of components. Micelles for oral delivery have not yet been extensively investigated in vivo, likely due to interaction of bile salts and other food components with these systems leading to low stability.[194] The bioavailability of drugs in micellar formulations may still be too low or too variable to merit clinical development.[206]

### 2.5.6 Liposomes

Oral delivery of liposomes has been investigated for the purpose of drug and protein delivery.[188] Liposomes consist of an inner aqueous core surrounded by a membrane. Membrane components can be composed of naturally derived phospholipids such as egg phosphatidylethanolamine or dioleoylphosphatidylethanolamine (DOPE), phosphotidyl choline or phosphotidyl inositol.[11] Oral liposomes have special requirements beyond intravenous liposomes due to the harsh gastrointestinal environment. Liposomes generally need to maintain stability in the gastrointestinal milieu and release their drug at the site of absorption. Liposomal systems can be unstable in the presence of bile

salts, phospholipids, lipases, acidic environments and other physiochemical and enzymatic barriers native to the gastrointestinal environment. Especially in the case of liposomes carrying small hydrophobic species that can easily precipitate in aqueous environments, the understanding of lipid metabolism and colloidal changes due to additional endogenous lipids can be important.[207]

In spite of these difficulties, liposomes for oral delivery have shown some promising results. The oral delivery of insulin using bile salt sodium glycocholate and soybean phosphatidylcholine as liposome forming agents was evaluated in rats.[208] These agents have both penetration enhancing and enzyme inhibiting effects. Sodium glycocholate liposomes showed a 5.7% and 11.4% insulin bioavailability in nondiabetic and diabetic rats, respectively. Hypoglycemic levels as low as 63% of control samples were achieved. Oral dosing of controls involving admixed liposomes and insulin (no encapsulated insulin) were done. No significant increase in blood insulin levels or hypoglycemic effect was observed for control admixed solutions.[208] This confirms the importance of the liposome as a protective carrier for insulin. Also of note is that liposomes of size 2μm showed no significant hypoglycemic effect or insulin bioavailability, confirming the importance of nanoparticle size.[208] This recent study highlights the positive effects of liposomes on oral drug delivery and represents a relatively high bioavailability compared to other studies with chitosan[95,119,209,210] and liposomes.[211] Mechanistic studies support the absorption of the liposome with cargo as opposed to free insulin solution. Higher bioavailability could be due to the better protective effect against proteolysis in the GIT.

PEGylated liposomes were also studied for oral delivery of human epidermal growth factor in rats.[212] Dipalmitoylphosphatidylcholine (DPPC) liposomes carrying recombinant epidermal growth factor showed a 2.5 fold increase in AUC using liposomal formulations over growth factor solution. Gastric healing of ulcers was modulated using these liposomal carriers.

Studies with PEGylated liposomes have been performed for oral vaccination purposes.[213] PEGylated liposomes were observed to cause an effective mucosal immune response. The effect of lipid ratio on antigenicity was analyzed. It was observed that a higher relative amount of phospholipid dose (12.5µmol vs. 5µmol) for encapsulation of the antigen protein led to a higher (1.7 fold) IgG response. On the other hand it was found that an IgA response was reduced by increasing the phospholipid dose.

Despite these results, most attempts to orally deliver vaccines using liposomes have ended in failure.[214] For example Orasomes™ were developed for oral delivery of drugs and also for targeting M-cells for vaccination purposes. These nanopreparations were initially promising based on the stable cross linking lipid components making them resistant to detergent disruption.[189] In vivo studies demonstrated increased uptake in M-cells through the use of UEA-1 (Ulex europaeus 1) mouse M-cell targeting protein,[215] but these systems did not advance to clinical trials.[216] The reasons why these systems have been unsuccessful are probably multifaceted. Insufficient loading, irreproducible manufacturing, difficulties in scale up and low uptake by M cells may be causes of the lack of translation.[188]

While we still await a successful clinical outcome from oral liposomal delivery of drugs or proteins, future research should be focused on increasing permeability and absorption into systemic circulation. Higher loading and longer release profiles may also be critical for increased drug delivery to the site of action. Stability is also of importance and related to both of these aims. New work in the area of archaeosomes (liposomes made from lipids extracted from archaebacterias) could help achieve these goals, as they are based on non-saponifiable lipids. Archaeosomes have shown 3.5 fold increased absorption of $^{99}$technetium-diethylene triamine pentaacetic acid (DTPA) in rats over conventional liposomes.[217] Other combination strategies using polymer coated liposomes could also aid in stability, release and drug loading goals.

### 2.5.7 Solid lipid nanoparticles

Solid lipid nanoparticles as drug delivery systems have been actively studied for many years.[218] These systems are similar to liposomes, but use solid lipids instead of liquid, create a homogenous hydrophobic core, and can maintain stability for sustained periods of time in vivo.[219]

These nanopreparations are generally composed of glyceride molecules manufactured easily through high pressure liquid homogenization or phase inversion temperature shifts.[220] These lipids are usually of varying chain length because single size lipids may form highly crystalline particles and expel drugs from the interior regions. The heterogeneity of the lipid chains has found to be conducive to drug loading.[218] Generally lipids such as caprylic acid or capric acid

can be used for these systems with PEG and small amounts of lecithin are added for stability. These compounds are currently used in oral, topical and parenteral administrations and are generally considered safe.[219–221]

Oral administration of solid stearic acid nanoparticles coated with poloxamer 188 (mean diameter 196.8 nm, ζ-potential -69mV) loaded with camptothecin showed increased absorption in vivo compared to camptothecin solution. Interestingly the solid lipid nanoparticle formulation displayed a double peak, presumably from the initial burst release and subsequent sustained release from the particle core. Delivery of all-trans retinoic acid (ATRA) has been investigated by oral gavage in male rats.[222] It was found that solid lipid nanoparticles increased ATRA oral bioavailability approximately 5 fold over ATRA solution. These studies provide the rationale for oral solid lipid nanopreparations for drug delivery.

Cyclosporin A has also been dosed orally in solid lipid nanoparticle formulations.[223–225] Microemulsions of cyclosporin have shown nephrotoxicity and variable bioavailability.[225] Attempts have been made to overcome these problems using nanoparticles loaded with 20% cyclosporin. The lipid Imwitor was used with Tagat S (PEG-30 glyceryl stearate) and sodium cholate to stabilize the nanoparticles. The solid lipid nanoparticle formulation was found to eliminate the initial peak in cyclosporin blood concentration and exhibited sustained blood concentrations.

Treatment of rats with insulin loaded solid cetyl palmitate lipid nanoparticles showed a hypoglycemic effect.[113] These nanoparticles prepared by

solvent emulsification-evaporation were 350 nm in diameter and had a 43% loading efficiency. Poloxamer 407 was used to stabilize the nanoparticles. After oral administration to rats, a hypoglycemic effect or area above the curve (AAC) was 484 versus 260 for insulin loaded nanoparticles, and insulin alone, respectively. The hypoglycemic effect lasted for 24 h and was significantly lower than insulin control.

Solid lipid nanoparticles formulated from glyceryl monostearate were used to encapsulate vinpocetine.[226] These nanoparticles were studied for drug release and particle stability. Vinpocetine, which is normally highly insoluble, was stable in these nanoparticles for up to a year. Tween 80 and poly(oxyethylene) hydrogenated castor oil were used to stabilize the nanoparticles, but were also found to enhance the oral bioavailability of the nanoparticles. The nanoparticle formulation increased the bioavailability of vinpocetine significantly. For a more exhaustive analysis of types of therapeutic agents included into solid lipid nanoparticles readers are referred to a relevant review.[219]

Mucosolvan™ (ambroxol) is the only solid lipid nanoparticle product that has reached the market to date for treatment of acute and chronic bronchopulmonary disease. (Boehringer-Ingelheim, Germany). Other products in this area, such as Rifamsolin™ which was in phase I clinical trials in 2005, appear to have been abandoned (AlphaRX now UMeWorld, Canada).[227] The reason for the lack of further development of some solid lipid nanoparticle formulations appears to be instability in the gastrointestinal tract.[228] Other problems associated with these systems could be the lack of sufficient

improvement of absorption to warrant clinical success. Future research into the use of solid lipid nanoparticle could focus on less degradable lipid formulations (such as the previously mentioned archaeosomes) and increased penetration enhancement.

### 2.5.8 Nanosizing of standard formulations

A large portion of both current drugs on the market and investigational pharmaceutical agents have low solubility in water.[22,225] Nanopreparations have been able to increase the solubility of hydrophobic drugs by increasing the surface area exposure of the solid formulation. Reducing the size of solid pharmaceutical ingredients can increase the dissolution rate and saturation solubility of drug. This can be of critical importance for low solubility drugs. The increase in saturation solubility of the drug creates a greater local concentration gradient across the intestinal epithelium aiding in absorption. This has been shown to increase the oral bioavailability of several formulations (in addition to creating novel intellectual property for drugs coming off of patent). The theoretical basis for this occurrence revolves around the Nernst-Brunner equation[229]:

$$\frac{dw}{dt} = \frac{D}{h} \cdot (\mathrm{S}(\mathrm{Cs} - \mathrm{Ct}))$$ .....(Equation 2.2)

where dw/dt: Dissolution rate (mg/s), S: Effective surface area of the solid drug ($cm^2$), Cs-Ct: Concentration gradient (mg/mL), Cs: Saturated concentration (mg/mL), Ct: Concentration of the solute at time t (mg/mL), h: Effective diffusion

layer thickness (cm), and D: Diffusion coefficient (cm/s). Dissolution rate is positively affected by increasing the surface area based on this equation. This can be achieved through reducing the size to nanodimensions. It has been observed that nanosizing can have a dramatic effect on the oral bioavailability of the drug in vivo. For example in an oral dosing of naproxen in rats the $AUC_{0–240}$ of the nanoparticle suspension was 125% that of the free solution.[230] Indeed, one of the first reports to analyze the effect of nanosizing a formulation on oral bioavailability showed a 50–80% increase in bioavailability of phenylbenzoylurea derivative HO-221 by decreasing the particle size from 17μm to 450 nm in rats and dogs.[231,232]

The demonstration of clinical successes using this solubilization of small molecules is the greatest of any oral nanopreparation category to date. A large number of pharmaceuticals have already been approved using the nanosizing technology (Table 2.6). Tricor sales alone have exceeded $1 billion, soundly placing this paradigm into mainstream pharmaceutical industry.[233] The nano formulation showed reduced variability between fed and fasted states for the delivery of fenofibrate and allowed reduction in the dosage level. A variety of other products have progressed to clinical trials for oral delivery using this technology.[45] Élan Pharmaceutical Technologies (now Alkermes, Ireland) has trademarked the methods used for creating nanosized pharmaceuticals as Nanocrystal™ while SkyPharma uses the term Dissocubes™.[31,33] These nanoparticles are produced in a top down approach, where larger particles are milled or homogenized to smaller particle sizes. Resins are usually 500μm beads

**Table 2.6.** Marketed oral nanopreparations[21,272]

| *Product Name* | *Drug* | *Technology* | *Company* |
|---|---|---|---|
| Rapamune® | Sirolimus | Nanosuspensions | Wyeth Pharmaceuticals/Élan |
| Megace ES® | Megesterol Acetate | Nanosuspensions | Par Pharmaceuticals/Élan |
| Emend ® | Aprepitant | Nanosuspensions | Merck/Élan |
| Tricor ® | Fenofibrate | Nanosuspensions | Abbott Laboratories/Élan |
| Triglide ® | Fenofibrate | Nanosuspensions | SkyePharma/Sciele |
| Avinza ® | Morphine sulfate | Nanosuspensions | King Pharmaceuticals |
| Focalin XR™ | Dexmethyl-phenidate HCl | Nanosuspensions | Novartis |
| Ritalin LA® | Methylphenidate HCl | Nanosuspensions | Novartis |
| Zanaflex® | Tizanidine HCl | Nanosuspensions | Acorda |

and milling can be completed within a few h. Difficulties of nanosizing formulations include stabilization of the nanoparticles after synthesis. Stabilization is necessary because of the high surface energy created by the small size. If this surface energy is not tailored to the right range, Ostwald ripening or agglomeration can occur. The optimal ratio of stabilizer is usually 20:1 to 2:1 and can include cellulosics, pluronics, polysorbates and povidones. Combinations of ionic and nonionic stabilizers are often used to decrease aggregation and Ostwald ripening. In spite of additional stabilizers the dispersion can be made into tablets, capsules and fast melts adding additional versatility to this approach.[10,33]

This approach is limited to those drugs that are dissolution rate limited since nanosizing itself does little to increase penetration of the intestinal epithelium. Other problems that still exist for nanosizing technology include the empirical method of screening excipient stabilizers for each drug and lack of miniaturized processes supporting discovery. Future work into novel mechanisms of synthesis, such as spray freezing into liquid or evaporative precipitation into aqueous solution could aid in reducing particle size below 100nm. Patents for this technology will soon end, opening the door for other companies to take advantage of nanosizing drug formulations for insoluble molecules.[233] Many small molecule drugs have yet to be tested for nanosizing effects, but the benefits may be well worth the cost.[10,234] Choosing a system optimal for oral delivery among the mentioned materials must be based on requirements in size, loading, charge, toxicity and cargo. Nevertheless the fraction absorbed observed

for PAMAM dendrimers in mice, has given rationale for their continuing investigation in oral drug delivery.[170,235] The fact that apparent permeability ($P_{app}$) generally decreases with increasing molecular weight, dropping to $<5\times10^{-6}$ cm/s at a molecular weight of 500 Da for poly(ethylene glycol), while 14 kDa PAMAM dendrimers have a $P_{app}$ of $>30\times10^{-6}$ cm/s in Caco-2 is promising.[167,236] Additional studies on the transepithelial transport of dendrimers in isolated animal and human intestine will aid in understanding the clinical potential of dendrimer oral drug delivery.

## 2.6 Conclusion

Oral administration of PAMAM dendrimers may be able to combine the advantages of polymeric drug delivery and simultaneously deliver drugs via the oral route. PAMAM dendrimers have been shown to enhance the solubility of low solubility drugs. Furthermore, PAMAM dendrimers have the ability to penetrate through the intestinal epithelium at high rates, making them optimal for oral dosing. The oral route of administration remains the preferred route for dosing of pharmaceuticals. Decreased stringency for production as well as increased patient quality of life are the driving forces behind manufacture of oral formulations.

Estimation of PAMAM dendrimer oral drug delivery is largely based on in vitro models such as Caco-2 which lack human like properties such as mucous, transport proteins, metabolic enzymes and tissue morphology. On the other hand, isolated tissue models have these properties and have been shown to be

predictive of human fraction absorbed, especially when using human isolated tissue. Likewise, isolated tissue models have higher throughput than in vivo models and do not require expensive clinical trials in order to test human intestinal transport.

Overall the goal of realizing dendrimer oral drug delivery may require the use of penetration enhancers to achieve high rate of absorption. While dendrimer penetration of the intestinal epithelium has shown to have damaging effects on the intestinal barrier, peptide based penetration enhancers have shown little effect on epithelial morphology. These enhancers have an ability to enhance macromolecular transepithelial transport. This has provided rationale for the use of peptide penetration enhancers in conjunction with PAMAM dendrimers to achieve a nontoxic oral drug delivery.

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# CHAPTER 3[1]

# TRANSEPITHELIAL TRANSPORT OF PAMAM DENDRIMERS ACROSS ISOLATED RAT INTESTINAL TISSUE

## 3.1 Introduction

Polymeric drug delivery can improve solubility, biodistribution and bioavailability of insoluble and highly toxic drugs.[1] Poly(amido amine) (PAMAM) dendrimers are a highly branched class of polymers that can increase solubility and intestinal permeability of drugs.[2–4] These versatile carriers have multiple surface groups which can be functionalized with imaging agents, drugs, labels and targeting ligands.[5–8] PAMAM dendrimers can also be surface-engineered to tune their toxicity and pharmacokinetic profiles, allowing them to be tailored for specific biomedical applications.[9,10]

PAMAM dendrimers penetrate the intestinal barrier *in vitro* and *in vivo,*

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[1] Note: Reprinted with permission from D. Hubbard, H. Ghandehari, D. J. Brayden, Transepithelial transport of PAMAM dendrimers across isolated rat jejunal mucosae in Ussing chambers, *Biomacromolecules,* 2014, 15, 8, 2889-2895. Copyright 2014 American Chemical Society.

suggesting a rationale for use in oral drug delivery.[11–19] The oral route of drug delivery has the distinct advantage of increased patient compliance, reduced risk of needle-borne infections and improved pharmacokinetic profiles compared to parenteral dosing.[20] Many chronic diseases including cancer treatments require years of regular injections, which could be circumvented if the drug was absorbed orally. Many other small molecules require injection due to their poor intestinal solubility and low or variable intestinal absorption, especially when the drug has a narrow therapeutic index. PAMAM dendrimers have shown to increase the intestinal permeability of camptothecin,[21] propanolol,[22] naproxen,[23] SN38,[24] and silybin.[25] This provides rationale for the development of PAMAM dendrimers as an oral drug delivery system for poorly absorbed drugs. In this chapter we evaluated the ability of PAMAM dendrimers to be absorbed through the isolated rat jejunal epithelium.

Caco-2 cell cultures have been used for the majority of evaluations of PAMAM dendrimer transport through the intestinal epithelium.[10,12–15,17,18,26,27] Such studies provided analysis of dendrimer intestinal penetration and toxicities. They revealed that dendrimer permeation is a function of the dendrimer generation, concentration, and incubation time.[18] These studies have also provided mechanistic insights into the routes *via* which PAMAM dendrimers can penetrate the intestinal epithelium. Previous work demonstrated that specific pharmacologic endocytosis inhibitors reduced the flux of G4 PAMAM dendrimers across Caco-2 monolayers.[17] Further work showed that PAMAM dendrimer transport was clathrin, dynamin and energy-dependent in Caco-2 cells. While this

indicates that endocytic mechanisms are involved in dendrimer transport, these studies also showed that dendrimers facilitated tight junction opening, as evidenced by occludin staining and increased mannitol transport.[10,13,14,28,29] Thus the route of dendrimer penetration across Caco-2 monolayers appears to be *via* a combination of the transcellular and paracellular route. Alternative data achieved in CD-1 mice indicated no increase in mannitol permeability or tight junction opening when PAMAM dendrimers were administered orally at concentrations greater than those used in Caco-2 cell cultures (e.g., G4-$NH_2$ (2.1mM), G3.5-COOH (7.7mM)).[21] This apparent discrepancy may be due to the differences between the models used. Cell culture models lack some of the properties that native tissues contain. In comparison to *in vivo* models, Caco-2 cell monolayers lack mucus layers, extracellular matrix proteins, supportive mixed cell populations, basement membranes and metabolic protein expression. Indeed, Caco-2 cell cultures can give a wide range of transport data based on differences between source,[30,31] selection pressure,[30] passage number,[32] and tissue culture conditions.[33–35] Caco-2 cells have been noted for their increased sensitivity to penetration enhancers, excessively high resistant tight junctions and increased indications of cytotoxicity upon exposure to enhancers compared to native isolated rat and pig intestinal tissue.[36,37] Thus, to account for such deficiencies in Caco-2 cells, we evaluated the mechanism of dendrimer permeability and toxicity in an isolated rat jejunal model in Ussing chambers.

The Ussing chamber model has been used to study the mechanisms of transport and toxicity of drugs across isolated intestinal tissue.[38] In this study we

utilized isolated rat intestinal epithelium to test PAMAM dendrimer transport due to its higher correlation to human jejunum effective permeability ($P_{eff}$) than Caco-2 cell cultures ($R^2$=0.95 vs. $R^2$=0.79, respectively).[39] The concentration, incubation time, and molecular weight of dendrimers used were chosen to exceed typical limits of cytotoxicity in Caco-2 cultures previously observed in our lab, in order to see if isolated tissue histology would similarly be affected. Due to the hypersensitivity of Caco-2 cell cultures to penetration enhancers, we hypothesized that a supratoxic concentration of PAMAM dendrimers would yield reduced evidence of toxicity in an isolated tissue model.[36,37,40] Thus, concentrations of 1.0 mM with an incubation time of 120 min with G4 dendrimers were used for this study as these have previously exhibited cytotoxicity to Caco-2 cells (Table 3.1).[10,14,15,18]

In addition the hypothesis that PAMAM dendrimers induce tight junction opening was explored in this study in order to reconcile results of previous mice and *in vitro* Caco-2 (Table 3.2).[14,21] Concentrations, incubation times and generations of PAMAM dendrimers were selected that have previously been reported to increase mannitol permeability in Caco-2 monolayers.[14] G3.5 and G4 PAMAM dendrimers of 1.0 mM size, incubated with tissue for 90 min increased mannitol permeability in Caco-2.[14,18] With this concentration, we planned to probe the differences between isolated tissue and Caco-2 models as influenced by PAMAM dendrimers. This study provides evidence for the role of isolated tissue studies in oral drug discovery and the limitations of Caco-2 cells for toxicity screening.

**Table 3.1.** Intestinal toxicity in the presence of dendrimers*

| G3.5 PAMAM Dendrimers | | | | |
|---|---|---|---|---|
| | 0.01mM | 0.1mM | 1mM | 10mM |
| 90 min | | -[a] | -[a] | +[a] |
| 120 min | -[b] | -[b] | -[c]-[e] | -[d] |
| 150 min | | +[a] | +[a] | +[a] |
| 180 min | | -[k] | -[k] | |
| 210 min | | +[a] | +[a] | +[a] |
| **G4 PAMAM Dendrimers** | | | | |
| | 0.01mM | 0.1mM | 1mM | 10mM |
| 90 min | | | +[f] | +[f] |
| 120 min | -[e]-[h] +[g] | +[e]+[g] | -[c]-[j] +[e]+[g] | |
| 150 min | | | +[f] | +[f] |
| 180 min | -[i] | +[i]+[k] | +[i] | |
| 210 min | | +[f] | +[f] | +[f] |

*(+) = Indication of toxicity (-) = No indication of toxicity

a. Caco-2 - LDH release[14], b. Caco-2, WST-1 assay[27], c. This study - Rat jejunum - Histology, Carbachol Response, d. Mice, 7.7mM dose - Histology, TEM[21], e. Caco-2 - TEM[15], f. Caco-2 - LDH release[13], g. Caco-2, WST-1 assay[18], h. Caco-2 - WST-1 assay[17], i. Caco-2 - WST-1 Assay[10], j. Mice, 0.9mM dose - Animal Wt., Blood Chemistry[9], k. Caco-2 - MTT assay.[29]

**Table 3.2.** Mannitol permeability in the presence of dendrimers in different bioassays

| G4 PAMAM Dendrimers | | | |
|---|---|---|---|
| | 0.01mM | 0.1mM | 1mM |
| 90 min | - | - | 8 fold[a] |
| 120 min | 2 fold[b] | - | 12 fold[a], 0 fold[c,d] |
| **G3.5 PAMAM Dendrimers** | | | |
| | 0.1mM | 1mM | 10mM |
| 90 min | 4 fold[e] | 6 fold[e] | - |
| 120 min | - | 0 fold[d] | 0 fold[c] |

a. 1:8 FITC modified[18], b. Caco-2[10], c. 2.1mM via gavage to mice[21], d. this study - isolated rat jejunum, e. Caco-2.[14]

Thus the aim of the studies described in this Chapter was to evaluate the intestinal permeability of PAMAM dendrimers G3.5 and G4 in isolated rat jejunum, using concentrations that probe the limits of toxicity in isolated tissue versus Caco-2 cell culture. Additionally, transepithelial transport of PAMAM dendrimers was monitored to explore the feasibility of dendrimer oral drug delivery for future biomedical use.

## 3.2 Materials

PAMAM dendrimers (G4.0 and G3.5) were purchased from Dendritech, Inc. (Michigan, USA). FlTC and FITC-dextran were obtained from Sigma-Aldrich (Dorset, UK). Acetone was obtained from VWR (Ireland). Carbachol was obtained from Calbiochem, Inc. (Massachusetts, USA). Disposable size exclusion PD-10 Columns were obtained from GE Lifesciences (Buckinghamshire, UK). $^{14}$C Mannitol (56.5 mCi/mmol) was obtained from Perkin-Elmer (USA). All other reagents were obtained from Sigma-Aldrich (Ireland).

## 3.3 Methods

### 3.3.1 Synthesis of FITC-labeled PAMAM dendrimers

FITC-dendrimer conjugates were synthesized using previous methods with some modifications.[15] Briefly, FITC was dissolved in acetone (<5mg/mL) and added to amine-terminated (G4.0) dendrimers at a ratio of 1:1.2, at pH 7.4 in PBS. The reaction proceeded overnight with stirring at room temperature and the product was then dialyzed for 24 h.

The carboxylic groups of G3.5 were activated with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC), and then tert-butyl N-(2-aminoethyl) carbamate (molar ratio 1:12:4) was added in PBS at a pH of 7.4. The reaction was then dialyzed for 24 h. The tert-butyl (Boc) protecting group was removed by adding 1 mL of trifluoroacetic acid to the dialysate and stirring for 4 h, followed by further dialysis (24 h, four water changes). These slightly amine-modified dendrimers were then reacted with FITC similar to G4.0 dendrimers above.

FITC conjugated dendrimers were fractionated by size exclusion chromatography using a Fast Protein Liquid Chromatography (FPLC) system to remove small molecular weight impurities. Fractions were taken from 161mL to 232mL elution volume. FITC conjugated dendrimers were fractionated using a XK 26/70 column packed with Superdex 200 prep grade media (GE Lifesciences, Buckinghamshire, UK) at a flow rate of 2.5mL/min of PBS (pH 7.4 PBS). They were then further dialyzed and lyophilized. The FITC-dendrimer conjugates were analyzed by FPLC to assess for small molecular weight impurities. FITC loading was quantified spectrophotometrically (Fig. 3.1).

### 3.3.2 Ussing chamber experiments

Isolated jejunal tissue was obtained from male Wistar rats (Charles River, UK) of weight 250-500g in accordance with the UCD Animal Research Ethics Committee policy on use of tissue postmortem. Rats were sacrificed by cervical dislocation, followed by immediate removal of the jejunum (up to 20 cm proximal

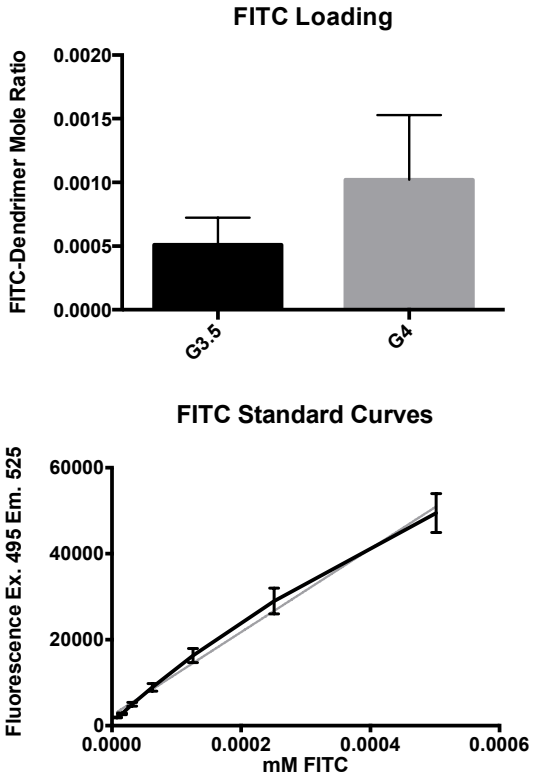


**Figure 3.1.** PAMAM dendrimers were evaluated for FITC loading following conjugation. Samples of G3.5 and G4 FITC conjugate were weighed and then dissolved in 100μL PBS pH 7.4. The samples were then read for fluorescence (λ ex./λ em. of 495/525 nm) on a fluorescent plate reader calibrated with FITC standard curves on the same plate (MD Spectramax Gemini). The FITC-loading was then quantified by calculating the concentration of FITC attached to the total mass of dendrimers. The molar ratio of FITC to dendrimer was quantified spectrophotometrically using a FITC standard curve (lower graph, grey line represents model fit, $R^2$=0.98) to interpolate the FITC molar ratio of dendrimer-FITC conjugate (upper graph) .

from cecum). Tissue was immediately immersed in fresh Krebs-Henseleit (KH) buffer maintained at 37°C, pH 7.4 and oxygenated with carbogen gas. Tissue was then opened along the mesenteric border and was pinned mucosal-side down on a corkboard. The external muscularis layer was then gently stripped away from the submucosa using a watchmaker's size 5 fine forceps leaving an intact epithelium with lamina propria. Tissue was mounted between the two halves of an Ussing chamber (World Precision Instruments, UK) with a 5mL bath volume each side, a gas air-lift system and an 0.63cm$^2$ exposed tissue area.[41] Chambers were bilaterally filled with fresh oxygenated KH buffer. Following mounting, mucosae were equilibrated in oxygenated buffer for 15 min followed by 30 min of voltage clamping in order to calculate transepithelial electrical resistance (TEER) values and to ensure that levels were above minimum acceptable values (30 Ω.cm$^2$).[42] Test probes were added to apical side of tissue and sampling occurred (200µL) every 20 min for 120 min from the basolateral side, and at 0 and 120 min from the apical side. The chamber volume was maintained on the basolateral side by replacing sample volume with fresh oxygenated KH buffer after each sampling point.

### 3.3.3 Apparent permeability ($P_{app}$) measurement

Permeability of FITC labeled-PAMAM dendrimers (G3.5, G4.0), FITC, and FITC-dextran (4kDa;10 kDa) were tested across mucosae. FITC-dextrans were used as macromolecular control markers for paracellular flux. 0.5 µCi of $^{14}$C-Mannitol was also added to the apical side of all experiments to serve as a

marker of paracellular permeability. Fluorescence was detected in samples using a spectrophotometer (λ ex./λ em. of 495/525 nm, MD Spectramax Gemini). Samples were then transferred to vials and mixed with 3 mL of scintillation cocktail (Ecoscint, National Diagnostics). Scintillation counting was performed on a Packard Tricarb 2900 TR (Perkin-Elmer, Ireland).

### 3.3.4 TEER measurement

Following permeability experiments, the electrogenic chloride secretory responses of mucosae were tested to ascertain retention of intestinal function. A cholinomimetic, carbachol, was added to the basolateral side of the chamber at concentrations from 0.1μM to 10μM. The change in short circuit current (ΔIsc) was measured relative to the baseline current.[43,44] Tissue was then gently removed and fixed in 10% buffered formaldehyde for 24 h in preparation for histological staining with haematoxylin and eosin (H&E) or alcian blue and neutral red (AB/NR).[45]

The potential difference (PD) and ΔIsc across the epithelial layer was monitored by Ag/AgCl electrodes using an EVC-4000 amplifier (WPI, UK) and Pro-4 timer (WPI, UK). 3M KCl solution in 3% agar (w/v) was used as an electrode bathing solution. Electrical signals were converted from analogue to digital using Powerlab® data acquisition unit. Data were recorded with Chart® software (AD instruments, UK) and TEER was calculated indirectly using Ohm's Law from the Isc and PD values. Voltage clamping to zero was performed using a cyclical 30-sec voltage clamp to 0 mV followed by 3 sec open circuit period

using the Pro-4 timer.

Apparent permeability coefficients ($P_{app}$) of fluorescent and radioactive compounds were calculated using the equation:

$$P_{APP}=(dQ/dt)/(C_o \times A) \quad \text{……..……(Equation 3.1)}$$

where dQ/dt is the rate of appearance of sample on the basolateral side, $C_o$ is the apical concentration and A is the exposed surface area of the tissue.

### 3.3.5 Statistical analysis

Permeability data were analyzed using GraphPad® Prism (version 6.0c). Analysis was performed using one-way analysis of variance (ANOVA) with Tukey's post-test (pooled variance). Statistics on TEER values was performed using a two-tailed Student's t-test comparing TEER at t=0 to the later time points. Values with $P<0.05$ were considered significant.

## 3.4 Results

FITC-labeled dendrimers were synthesized and fractionated to remove free FITC prior to testing in Ussing chambers. Purification by FPLC resulted in removal of small molecular weight peaks occurring after 232 mL elution volume on the XK 26/70 column (Figure 3.2).

$P_{app}$ values for FITC-labeled PAMAM dendrimers through isolated rat jejunum were obtained in Ussing chambers. The $P_{app}$ of FITC-G3.5 PAMAM

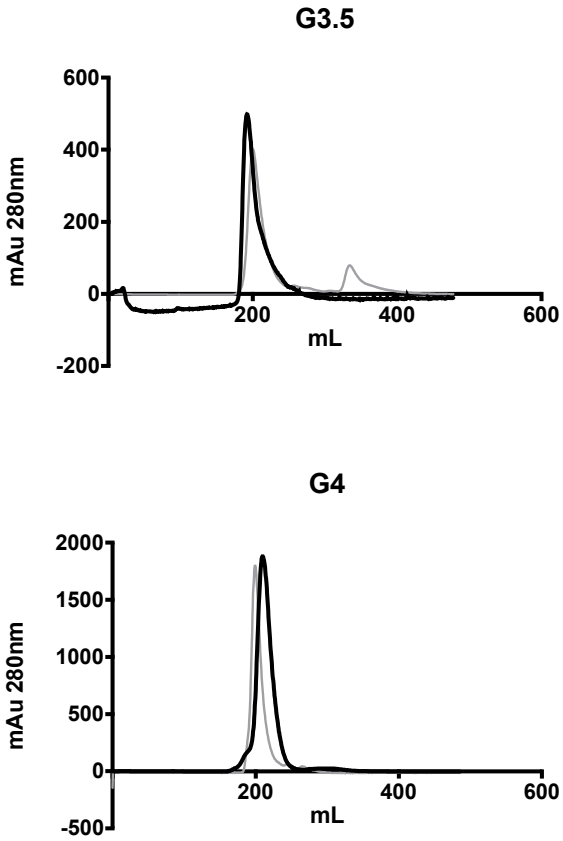


**Figure 3.2.** Size exclusion chromatograms of G3.5-FITC and G4-FITC conjugates before and after fractionation. Grey line = before fractionation, Black line = after fractionation.

G4.0 dendrimers was not statistically increased compared to free FITC, although there was a trend. Importantly, the $P_{app}$ of FITC-dextran (both 4kDa and 10 kDa) was not different from that of free FITC (Figure 3.3), so it is not the case that conjugation to any molecule increases the FITC $P_{app}$ *per se*. The [$^{14}$C]-mannitol $P_{app}$ was not significantly increased in the presence of either of the two dendrimer conjugates compared to untreated (Figure 3.4).

The average basal TEER for jejunal segments was 57±20 Ω.cm$^2$ (n=45). This is consistent with previously reported rat jejunal TEER values.[40,44,46] TEER values of the control, G4.0 dendrimer and FITC dextran treatments were significantly reduced after 40 to 60 min in Ussing chambers compared to baseline TEER at t=0. The TEER of the G3.5 treatment was observed to drop much more rapidly, reaching significantly reduced levels at t=5 min and onward ($P<0.05$). Decreases in TEER were not reflected in increased $P_{app}$ of mannitol (Figure 3.4), so the relevance of transient TEER decreases to overall paracellular permeability is questionable (Figure 3.5).

The electrogenic chloride transport secretory response to carbachol showed similar concentration-dependent, large $I_{SC}$ increases in jejunal mucosae exposed to apical additions of both unconjugated dendrimers and FITC dextrans for 120 min (Figure 3.6). Carbachol-stimulated $I_{SC}$ increases in the presence of dendrimers were similar to untreated controls and demonstrated that tissue secretory function was retained.

Histological evaluation at 120 min postmounting in chambers showed mild edema in all samples including untreated controls, likely due to the severance of

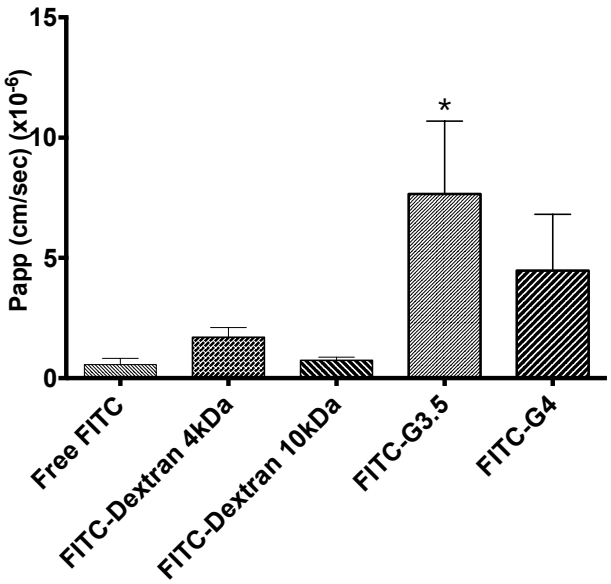


**Figure 3.3.** $P_{app}$ of FITC-PAMAM (1.0mM), FITC-dextran 4kDa (0.625mM), FITC-dextran 10kDa (0.25mM) and free FITC (0.02mM) across isolated rat jejunum. FITC-G3.5 dendrimers had significantly increased $P_{app}$ compared to free FITC (asterisk, $p < 0.05$).

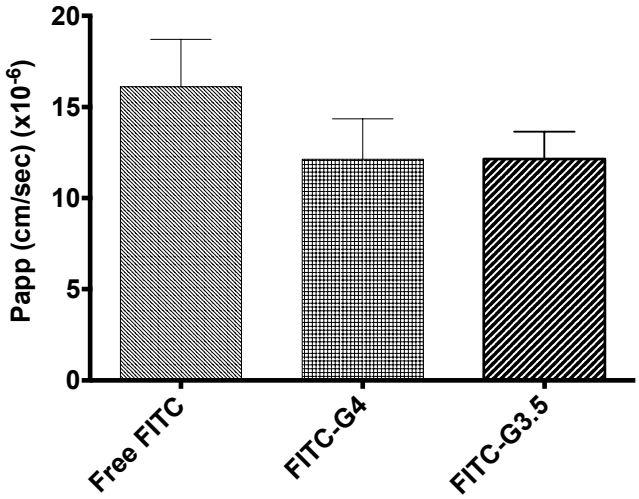


**Figure 3.4.** $P_{app}$ of [$^{14}$C]-mannitol through isolated rat jejunum. No significant difference was observed for G4.0 and G3.5 dendrimer (1.0mM) treatments versus free FITC, indicating no enhanced paracellular transport in the presence of either dendrimer.

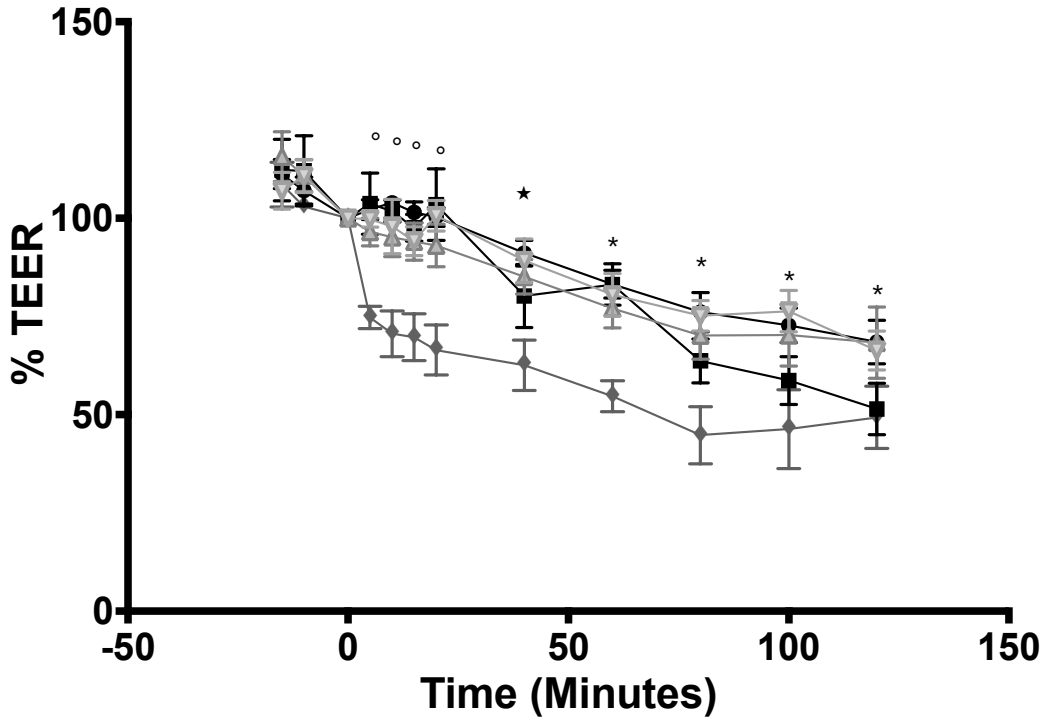


**Figure 3.5.** Percent TEER changes of isolated jejunal tissue. Control (□), G4 dendrimers 1.0mM (□), G3.5 dendrimers 1.0mM (□), FITC-dextran 4kDa (Δ), FITC-dextran 10kDa (∇). Percent TEER values were calculated as a percentage of the initial TEER at t=0 in each group. Significant differences from TEER of each individual group at t=0 are marked with open circles (G3.5 dendrimers), star (control, G3.5 dendrimers, FITC-dextran 4kDa), and asterisk (all groups).

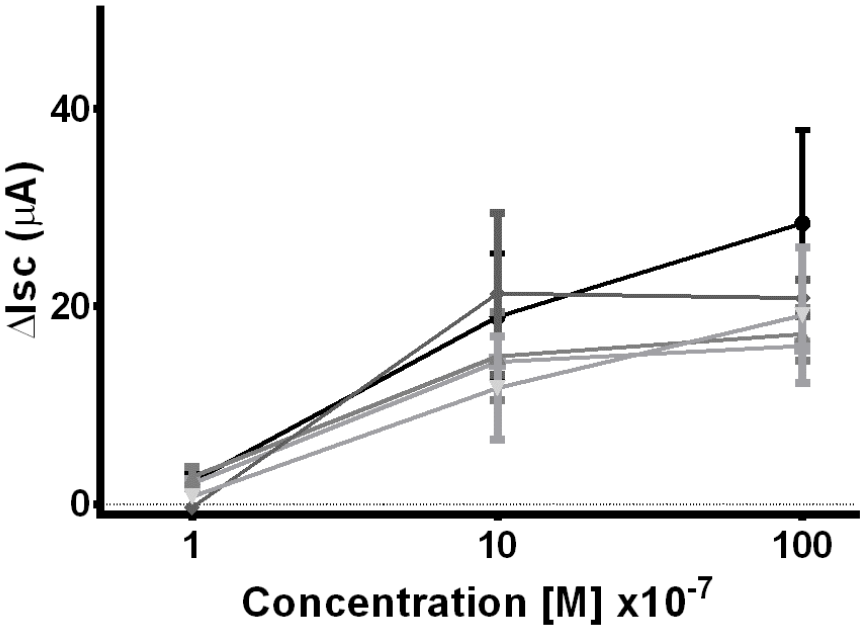


**Figure 3.6.** $\Delta I_{SC}$ response to basolateral additions of carbachol to jejunal mucosae. No significant difference in response was observed for test groups. Control (□), G4 dendrimers 1.0mM (□), G3.5 dendrimers 1.0mM (□), FITC-dextran 4kDa (∇), FITC-dextran 10kDa (Δ).

dendrimers was significantly increased over that of free FITC. The $P_{app}$ of FITC-lymphatic drainage, but there was no significant membrane disruption due to dendrimer treatments (Figure 3.7). All tissues therefore showed an intact barrier, consistent with the retention of secretory ion transport capacity.

The absence of small molecular weight FITC of the FITC-dendrimer conjugate was monitored through size exclusion chromatography on PD-10 columns after each experiment. FITC-dendrimers collected from the basolateral chamber remained stable, with no appearance of the free label peak (30mL elution volume) compared to the dendrimer peak (6mL elution volume). This signifies that detected fluorescence on the basolateral side of the chamber was not due to free FITC cleaved from the dendrimer, but rather due to the FITC-labeled dendrimer. This result is critical to validating the stability of the conjugate during the 120 min flux period (Figure 3.8).

## 3.5 Discussion

PAMAM dendrimers have shown the capacity to permeate the small intestinal epithelium, and to increase the solubility of co-presented drugs *in vitro* and *in vivo*.[2] Their size and surface functionality makes them capable of a variety of biomedical functions.[3] The potential for PAMAM dendrimers in oral drug delivery has been validated in cell culture models and in animals.[9,11–14,21] It is remarkable that selected PAMAM dendrimers penetrate the rat small intestinal epithelium in spite of their large molecular weight, complex macromolecular structure and hydrophilic nature as shown here. In particular, G3.5 dendrimers

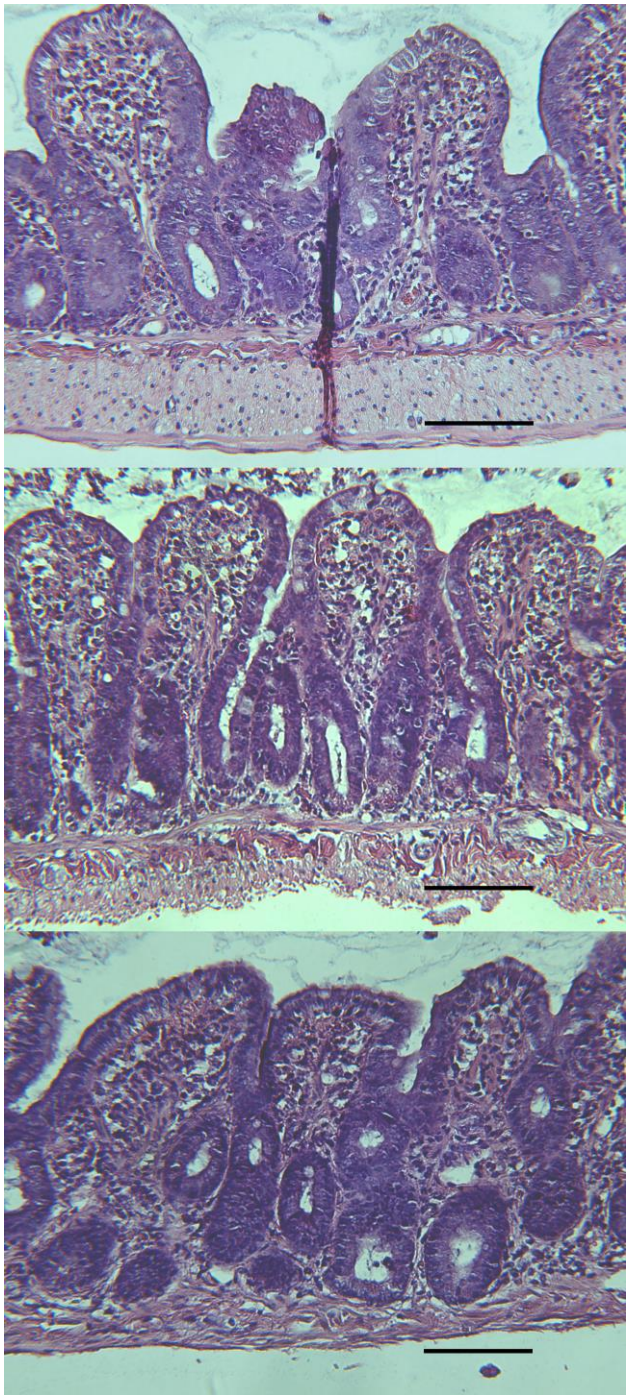

**Figure 3.7.** H&E staining of isolated jejunal tissue after 120 min incubation in Ussing chambers. No difference in histology was observed between controls and dendrimer-treated tissue. 1mM G3.5 dendrimer (upper), 1mM G4 dendrimer (middle), Control (lower). Scale bar = 100μm for all figures.

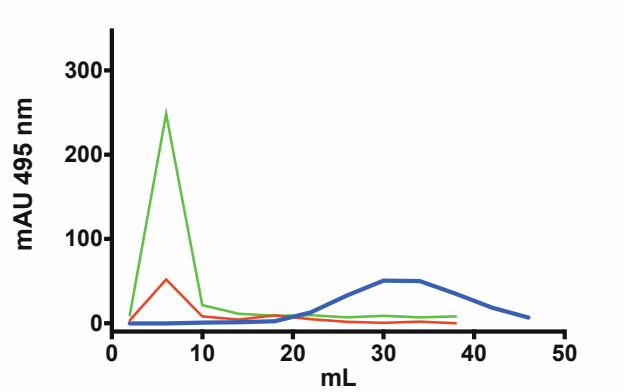


**Figure 3.8** The absence of free FITC during the Ussing chamber studies was evaluated by use of size exclusion chromatography on PD-10 columns after each experiment. Basolateral solutions were removed after the experiment and stored in 4°C until running on PD-10 columns. Each column was used one time to test for small molecular weight free FITC. Columns were washed prior to use with 12mL of PBS pH 7.4. 500µL of the basolateral solution was loaded onto the column followed by 1.5mL PBS. The eluted void fraction was collected (2mL) followed by 4mL fractions thereafter. Samples were collected into spectrophotometer cuvettes and analyzed for absorbance at 495nm (Pharmacia Biotech, Ultraspec 200 UV/Vis), as fluorescence signal was too weak to detect following dilution on the column. Blank elution solvent (PBS pH 7.4) was used to zero the absorption signal prior to each sample analysis and after each analysis to affirm that there was no drift in absorption signal. Stability of the FITC-dendrimer conjugates was monitored by size exclusion chromatography (PD-10) following fluxes across jejunal mucosae. FITC labeled dendrimers showed no peaks corresponding with free FITC at 120 min as detected by absorbance at 495nm (Free FITC – blue, G4 dendrimers – red, G3.5 dendrimers – green).

permeated the rat jejunum very well compared to free FITC, as indicated by a $P_{app}$ in excess of $7x10^{-6}$ cm/s. Although the probes would still be classified into BCS class III for low permeability/ high solubility molecules based on the results of this study, such a $P_{app}$ value is associated with an *in vivo* human $f_{ad}$ of 40-60%.[47] Indeed, anionic G6.5 PAMAM dendrimers that have a molecular weight 8 fold larger than the probes in this study have been observed to have an $f_a$ of 9.4% in mice.[11] Since larger molecules are generally more slowly absorbed than smaller ones, it is reasonable to expect an $f_a$ of 40-60% for the lower generation dendrimers studied here, but this could be argued based on the lower number of functional groups interacting with tissues in small generations. Future clinical application of PAMAM dendrimers rests upon careful evaluation of these parameters. The permeability of G3.5 and G4 dendrimers in Caco-2 cells has been previously studied in Caco-2 cell cultures and isolated tissue, facilitating comparison of results obtained from isolated tissue and cell culture models.[10,16,18] Kolhatkar et al. observed G4 dendrimers with a $P_{app}$ $1.5\times10^{-6}$ cm/s in Caco-2 cultures at 120 min incubation time.[10] This value of $P_{app}$ was much less than the value of $P_{app}$ ($4.47\times10^{-6}$ cm/s) we observed in this study, but the apical concentration was also 100 fold less. Kitchens et al. observed a $P_{app}$ value for G4 dendrimers ranging from $20\text{-}35\times10^{-6}$ cm/s at 1.0mM for 60-120 min incubation times.[18] These results far exceed the values obtained in our study, but the dendrimers used in their study had a ratio of dendrimer to FITC of 1:8, potentially altering the physiochemical properties (the dendrimers in this study had a dendrimer FITC ratio of 1:1.2).[18]

G3.5 PAMAM dendrimer $P_{app}$ values in previous studies were less variable in comparison (Table 3.1). Results from multiple Caco-2 studies observed $P_{app}$ values ranging from $2.5\times10^{-6}$ cm/s at 0.1mM to $6\times10^{-6}$ cm/s at 1mM and 120 min timepoints.[16,18] The values of $P_{app}$ for G3.5 dendrimers obtained in this study were in the same range as previous results from Caco-2. These results add to the debate on whether hydrophilic macromolecules and nanoparticles can penetrate the intestinal epithelium.[48,49] Similar molecular weight dextran molecules (4 and 10kDa) did not cross the intestinal barrier to the same extent as the G3.5 (12.9kDa) and G4 (14kDa) dendrimers, indicating the unique physiochemical characteristics and structure of dendrimers facilitate their higher than expected transepithelial transport.[12]

In an initial study Wiwattanapatapee et al. evaluated the transepithelial transport of G4 and G3.5 PAMAM dendrimers in everted rat intestinal sacs.[19] This study compared the endocytic index (EI) of the dendrimers (ng dendrimer transferred/mg intestinal tissue protein), bovine serum albumin (BSA) and other polymers. G3.5 and G4 dendrimers were noted for their higher EI than BSA and poly(vinyl pyrrolidone) polymers. Comparison to this study is not facilitated by the nature of the permeability data obtained (EI vs. $P_{app}$). Although the permeability data in that study were not entirely linear, this study indicated that PAMAM dendrimers penetrate the intestinal epithelium.[19]

The secondary aim of this study was to compare mechanistic information obtained from mannitol permeability in this study to previous results obtained in Caco-2 models. Interestingly, mannitol transport did not increase when tissues

were exposed to 1mM G3.5 and G4 PAMAM dendrimers. These results are in contrast to previous studies in Caco-2 where increased transport of mannitol in the presence of 1.0 mM G3.5 and G4 dendrimers was observed. G3.5 dendrimers at 120 min were observed to increase mannitol $P_{app}$ 6 fold, while G4 dendrimers at 120 min incubation time showed a 12 fold increase (Table 3.2).[14] This discrepancy may be due to the differences between isolated tissue models and Caco-2 cell culture. Isolated rat jejunal mucosae contain properties that map to the human jejunum.[39] These include mucus layers, extracellular matrix proteins, host enzyme levels,[47] supportive cells and basement muscle layers.[50–52] Lacking mucus and supportive cells underlying the epithelial barrier, Caco-2 cell cultures may be sensitive to PAMAM dendrimer induced mannitol permeability enhancement compared to rat jejunal mucosae. Other studies have noted the increased sensitivity of Caco-2 cells to penetration enhancers, compared to isolated tissue.[36,37] A recent study in CD-1 mice orally gavaged with G3.5 or G4 dendrimers formulated with [$^{14}$C]-mannitol also showed no induction of paracellular transport of mannitol confirming the results of this study.[21] Since mannitol is an indicator of tight junction opening and enhanced paracellular transport, this suggests that PAMAM dendrimers do not increase paracellular transport in isolated tissue and oral gavage *in vivo,* at the concentrations, generations, surface modifications and incubation times studied.

The alternative possibility would be transcellular route via an endocytic pathway. In parallel with the tight junction route, it has been confirmed in Caco-2 cells where $P_{app}$ of radiolabeled G4 was observed to decrease in the presence of

endocytic inhibitors.[17] Further work showed a decrease in G3.5 PAMAM $P_{app}$ during incubation with clathrin inhibitor (mondansyl cadaverine) or dynamin inhibitor (dynasore). The caveolin inhibitor, genistein, did not have a significant effect, indicating that transport across the membrane may depend primarily on clathrin-mediated transcytosis.[28] The effect of PAMAM dendrimers on the viability of epithelial layers was compared to controls. G3.5 and G4 PAMAM dendrimers of 1.0 mM concentration retained functional electrogenic chloride secretory pathways after 120 min incubation, indicating a lack of apical membrane disruption. Also, histological evaluation of the tissue showed intact villous structure. The signs of toxicity differ significantly in comparison with Caco-2 cell culture studies. In Caco-2 studies significant toxicity was observed at concentrations >0.1 mM and >90 min incubation times for G4 PAMAM dendrimers[13,15,18] and >1 mM and >150 min incubation times for G3.5 dendrimers, respectively.[14] Indications of reduced proliferation,[10,17,18,28] mitochondrial damage,[29] and cellular membrane damage[13,14] have been apparent. These effects were dependent on concentration, incubation time and assay as reviewed in Table 3.1. Suffice to say that the results of this study concord with *in vivo* studies performed in CD-1 mice that showed no toxicity for both PAMAM generations by TEM, morphological evaluation of microvilli as well as histological evaluation of intestinal epithelium.[21] The concentrations used in the *in vivo* study were actually higher than those in the present one. Similar oral studies have shown no signs of toxicity in CD-1 mice when dosed at 300mg/kg for G4 and G3.5 dendrimers.[53] More studies in the area of toxicity are needed to

understand the upper limit of safe oral PAMAM dendrimer administration. These studies give rationale for the potential use of PAMAM dendrimers to carry poorly bioavailable drugs across the intestine to their site of action. Future clinical application of PAMAM dendrimers rests upon careful evaluation of their permeability and toxicity profile.

## 3.6 Conclusion

The elevated transport of the G3.5 PAMAM dendrimers through isolated tissue gives rationale for dendrimer usage in oral drug delivery. The high permeability may indicate that PAMAM dendrimers can carry payloads into systemic circulation (such as poorly permeable and poorly soluble drugs (BCS Class IV)). PAMAM dendrimers' transport through isolated intestinal epithelia was greater than controls and greater than similar size FITC-labeled dextrans. Our results show minimal indication of toxicity to the epithelial barrier when treated with PAMAM dendrimers at concentrations of 1.0mM. This provides evidence that PAMAM dendrimers may be able to be dosed at nontoxic concentrations, yet still penetrate to a sufficient extent for increasing drug absorption. Payloads could potentially be delivered as a mixture or as conjugated moiety. Future work should be aimed at further understanding of the physiochemical characteristics that promote increased transepithelial transport and specific therapies for oral drug delivery. Results of this study indicate the potential of PAMAM dendrimers as an oral drug delivery system.

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# CHAPTER 4

# REGIONAL MORPHOLOGICAL DIFFERENCES AND TRANSPORT OF PAMAM DENDRIMERS ACROSS ISOLATED RAT INTESTINAL TISSUE AND CACO-2 MONOLAYERS

## 4.1 Introduction

Polymeric drug delivery has had a significant impact on clinical medicine, specifically by increasing drug circulation half-life, enhancing water solubility and reducing nonspecific uptake of drugs by nontarget organs.[1] With two polymeric drugs (Pegfilgrastim and Glatiramer acetate) amongst the 10 top selling drugs in the U.S. for 2014, the potential for polymeric systems to benefit the landscape of clinical biomedicine is tremendous.[2]

Polymeric systems have limited oral bioavailability.[3,4] Oral drug delivery is preferable over intravenous injections due to the ease of treatment, reduction in medical personnel required to administer the drug and reduction in patient time in hospitals. Additional factors that make oral delivery preferable include the reduced risk of needle borne infections and the improved pharmacokinetic profile of oral drugs versus bolus intravenous injections.[5] These aspects provide rationale for a focus on improving the oral bioavailability of polymers associated

with therapeutics.

Poly(amido amine) (PAMAM) dendrimers are a class of hyperbranched polymers with nanoscale dimensions and potential clinical application in oral drug delivery.[4,6] Dendrimers have multiple surface functional groups making them ideal for attachment of imaging agents, targeting ligands and therapeutic moieties.[7–12] Hydrophilic dendrimers can improve solubility of poorly soluble drugs.[13] PAMAM dendrimers have been observed to traverse the Caco-2 cell monolayers and enhance the permeability of drugs.[14–16] Kitchens et al. observed that generation 4 (G4) PAMAM dendrimers had a Papp in excess of $15\times10^{-6}$ cm/s in Caco-2 cell monolayers.[15] D'Emanuele et al. observed that G3 PAMAM dendrimers could enhance the transepithelial transport of propanolol across Caco-2 cell monolayers and significantly reduce its efflux by P-gp transporters.[17] The mechanism of dendrimer transport appears to be *via* a dynamin-dependent endocytosis and enhancement of paracellular permeation across epithelial barriers.[18,19] Specific CaMPKII inhibitors (KN62) have been observed to decrease dendrimer-induced tight junction opening (as reflected by increased [$^{14}$C]-mannitol $P_{app}$) in the presence of G3.5 dendrimers, thus implicating the CaMPKII molecular mechanism in dendrimer penetration in Caco-2 monolayers.[19] Other studies have noted the ability of PAMAM dendrimers to form perforations in plasma membranes (5-40nm in size), giving rationale for their interactions with lipidic membranes.[20] These mechanistic studies implicate dendrimers as having a specific charge-dependent effect on epithelial barriers that enables them to penetrate the intestinal epithelium by both paracellular and transcellular routes.

Significant in vivo evidence exists for the potential of PAMAM dendrimers to permeate intestinal tissue.[21] G6.5 PAMAM dendrimers have shown an oral fraction absorbed of 9.4% in mice.[22] In vitro studies with everted rat intestinal sacs have noted an endocytotic index for PAMAM dendrimers, which was higher than that of bovine albumin, *N*-(2-hydroxypropyl)methacrylamide, and N-vinylpyrrolidone-co-maleic anhydride.[23] Studies in our lab have noted the capacity of PAMAM dendrimers to increase the absorption of drugs both mixed with, or covalently attached to the dendrimer in vivo and in vitro.[6,24] Further investigations with rat isolated jejunal mucosae noted the higher $P_{app}$ of G3.5-fluorescein isothiocyanate (FITC) labeled dendrimers compared to free FITC.[25] This body of evidence gives rationale for the development of PAMAM dendrimers as oral drug delivery carriers, since they appear to be both transported across epithelial barriers and to enable absorption of associated pay-loads.

PAMAM dendrimers may have therapeutic potential, but the toxic interaction with the intestinal epithelium has also been noted.[3] Previous work in Caco-2 cell monolayers and intestinal tissues showed that positively-charged dendrimers disrupted cell membranes (as indicated by lactate dehydrogenase (LDH) release and morphological damage) compared to neutral- or negatively-charged dendrimers.[14,15,25–27] The cellular damage observed was incubation time- and concentration dependent.[14,26] LDH was released following exposure to 0.1mM G4 or G3.5 dendrimers in Caco-2 monolayers for 210 min.[14,26] Higher concentrations of G3.5 and G4 induced LDH release at earlier time points, 150 min and 90 min. On the other hand, oral dosing studies performed in CD-1 mice

dosed at 1000mg/kg PAMAM G3.5 dendrimers (~7mM intestinal lumen concentration) showed no intestinal morphology damage.[6] Consistent with that, no significant signs of histopathology were observed in isolated rat jejunal mucosae after 120 min incubation with 1 mM G3.5 and G4.[25] This trend led us to conclude that Caco-2 cell monolayers appear to be more sensitive to dendrimer - induced permeation enhancement and damage than isolated tissue or in vivo rodent models. Indeed, increased sensitivity of Caco-2 monolayers to penetration enhancers has been observed previously.[28,29] The discrepancy between the toxic concentration in cell culture and animal models has led us to re-evaluate the potential adverse effects of PAMAM dendrimers in isolated tissue models using Ussing chamber in order to help establish safe dose levels for PAMAM dendrimer oral drug delivery.

In addition to the maximum tolerated concentration of dendrimers by tissue mucosae, this study also sought to assess the regional transport of PAMAM dendrimers in rat colonic and jejunal mucosae, and to compare permeability $P_{app}$ values between Caco-2 and isolated mucosae. Previously, we established the $P_{app}$ of G3.5 and G4 across rat jejunal mucosae, but the colonic transport was not carried out.[25] Many penetration enhancers have significantly increased effects in colonic epithelium compared to jejunal tissues.[30] For this purpose we also explored the $P_{app}$ of G3.5 and G4 in colonic mucosae to assess their native ability to traverse the intestinal epithelium and serve as a drug delivery vehicle.

## 4.2 Materials

Fluorescein isothiocyanate (FITC) (cat. no. F7250), fluorescein isothiocyanate dextrans (cat. no. FD10S**)** and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) were obtained from Sigma-Aldrich. PAMAM dendrimers (G3.5-COOH and G4.0-$NH_2$) were obtained from Dendritech Inc. (Michigan, USA). $^{14}C$ mannitol (0.1mCi/mL in sterile water) was obtained from American Radiolabeled Chemicals (Missouri, USA). Gases were obtained from AirGas Corp (UT, USA). Cells were purchased from American Type Culture Collection (Rockville, MD). All other reagents were obtained from VWR.

## 4.3 Methods

### 4.3.1 Synthesis of FITC-labeled PAMAM dendrimers

FITC labeled conjugates were prepared as reported previously.[15,25] Briefly G3.5 dendrimers were dried from methanolic solution and dissolved in PBS pH 7.4. Ethylenediamine (ED) was conjugated to G3.5 dendrimers using a feed ratio of 1:4 dendrimer to ED. EDC was added to G3.5 dendrimers in PBS at 0°C for the first 30 min followed by the addition of ED. The reaction was allowed to stir overnight, followed by the addition of FITC at a feed ratio of 1:1.2, PAMAM dendrimer to FITC to complete the conjugation. G4 PAMAM dendrimers were mixed with FITC at a similar feed ratio in PBS to conjugate the FITC. All conjugates were then dialyzed for 48 h against deionized (DI) water. The final product was purified via fast protein liquid chromatography (GE life sciences) using an XK 26/70 column packed with Superdex 30 media at a flow rate of

2.5mL/min using PBS pH 7.4 as the eluent. The final purified compounds were dialyzed against DI water and lyophilized. The conjugates were characterized for absence of free FITC using Superose 6 column at a flow rate of 1 mL/min. FITC loading was assessed by spectrophotometry (Figure 4.1 and 4.2).

### 4.3.2 Caco-2 monolayer permeability

Caco-2 cell cultures were prepared according to previously published methods.[31] In brief, cells (passage 6-16) were grown at 37°C under a 5% $CO_2$ atmosphere in air with 95% relative humidity using Dulbecco's Modified Eagles's Medium with 10% fetal bovine serum (FBS). The media was changed every two days until 90% confluence was attained. Cells were passaged at least twice using trypsin-like enzyme (Gibcolife, NY, USA) before seeding onto Transwells®. Cells were seeded on 24-well polyester Transwells® (Sigma Aldrich, cat. no. CLfigure470-48EA) at a density of $2.6\times10^6$ cells/$cm^2$ and grew for 21-29 days before use. Transport medium consisted of Hank's balanced salt solution (HBSS) supplemented with 10 mM *N*-(2-hydroxyethyl)piperazine-*N*´-Poly(Amidoamine) (2-ethanesulfonic acid) hemisodium salt (HEPES) buffer (pH 7.4).

Transport experiments were carried out with 60 min of incubation with transport medium, prior to treatments. At t=0 min, the transport buffer was decanted and treatment was added to the apical side. FITC-G3.5 and G4 dendrimers were added at 0.1mM concentrations in the transport buffer. Monolayers were then incubated at 37°C on an orbital shaker at 350 RPM (G76, New Brunswick, NJ, USA). Aliquots were sampled at 200 µL every 30 min from

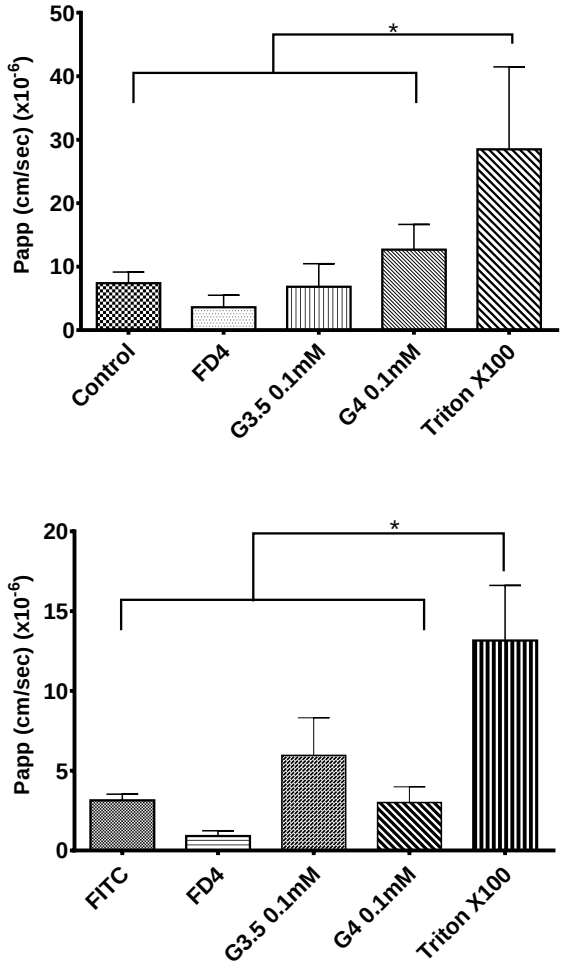


**Figure 4.1.** $^{14}C$ mannitol permeability across Caco-2 cells in the presence of dendrimers (top panel) and permeability of FITC-labeled dendrimers across Caco-2 cells (bottom panel). The permeability of fluorescently labeled G3.5 dendrimers trended higher than free FITC (a small molecular weight control), while the permeability of G4 dendrimers was similar to free FITC. 4kDa FITC-labeled dextrans (FD4) were tested to compare to another inert macromolecule. FD4 had a permeability that trended lower than dendrimers and controls. Mannitol permeability showed no significant change compared to control. All results were statistically different from the positive control ($p<0.05$, $n=3-7$).

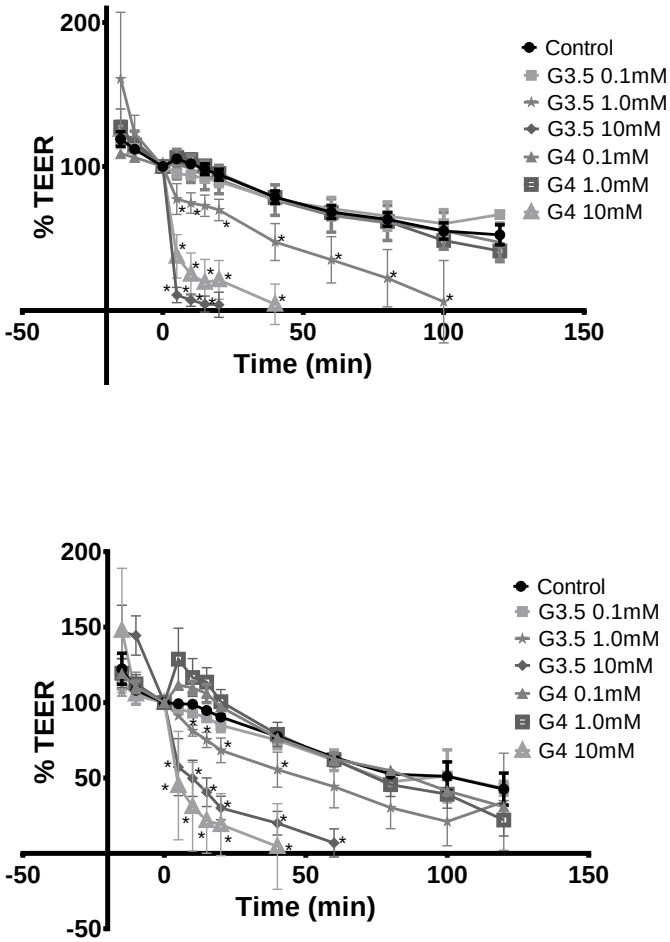


**Figure 4.2.** Dendrimer effect on percent transepithelial electrical resistance (% TEER) values in isolated rat intestinal tissue. Top panel: colon. Bottom panel: jejunum. PAMAM dendrimers had a concentration dependent effect on rat jejunal and colonic TEER. 0.1 mM concentration of dendrimers did not reduce TEER compared to control in colonic and jejunal tissue. 1.0 mM G3.5 dendrimers had a statistically significant reducing effect on TEER in both colonic and jejunal epithelium, while 1.0 mM G4 dendrimers had no such effect. 10 mM concentration of dendrimers immediately caused a reduction in TEER which lasted for the duration of the experiment in both types of tissues (n=3-12). * signifies $p<0.05$ compared to control.

the basolateral side starting at t=0 for 120 min and the volume was replaced by warm oxygenated buffer. Fluorescent samples were quantified using an excitation wavelength of 495 and emission wavelength of 525 on spectrophotometer (Spectramax M2, Molecular devices, CA, USA). All experiments included 5 µL of $^{14}$C-mannitol as an internal control, which was quantified using liquid scintillation (LS-6000IC, Beckman, CA, USA).

### 4.3.3 Ussing chambers

Animals were used according to the University of Utah Institutional Animal Care and Use Committee (IACUC) and the American Board of Veterinary Medicine guidelines. Female SAS-Sprague- Dawley rats were obtained from Charles River Labs (NY, USA) and housed in animal facilities on a 12-h light dark cycle with food and water *ad libitum*. Rats were used with a body weight range of 224-300 grams. Animals were euthanized *via* $CO_2$ asphyxiation followed by immediate dissection and removal of jejunum (approximately 20cm proximal to the cecum) or colon (approximately 2-3cm distal to the cecum).[32,33] Tissue was immediately immersed in ice cold oxygenated Krebs-Hensleit (KH) buffer at pH 7.4 and all further dissection was performed in a custom-designed oxygenated tissue dissection bath. Tissue was opened along the mesenteric border and pinned apical side down inside the mounting bath. The external muscularis layer was removed using No. 5 forceps and the intact epithelium was mounted in Easy Mount Ussing chambers with P2304 inserts (Physiologic Instruments, CA, USA) with a surface area of 0.3 cm$^2$. Chambers were filled with 5mL Krebs-Hensleit

buffer gassed with 95% $O_2$/5% $CO_2$ and equilibrated for 45 min at 37°C prior to treatments.

At time t=0 min, solutions containing FITC- or unlabeled dendrimers were added to the apical side of tissue. Dendrimers were added at concentrations of 0.1, 1.0, or 10mM for non-labeled and at 1.0mM concentrations for FITC labeled. All experiments included 5µL of $^{14}$C-mannitol as an internal control. The apical and basolateral side of the chambers were sampled at t=0 min and volume was replaced with 37°C, oxygenated KH buffer to maintain the volume at 5mL. The apical side was sampled at t=0 and 120 min while the basolateral side was sampled at t=0 min followed by 20 min intervals for 120 min. Samples were quantified as above.

The apparent permeability coefficients ($P_{app}$) of radioactive and fluorescent compounds was calculated using the equation:

$$P_{APP}=(dQ/dt)/(C_o \times A) \quad \text{.....(Equation 4.1)}$$

where dQ/dt is the rate of appearance of sample on the basolateral side, $C_o$ is the apical concentration and A is the exposed surface area of the tissue. Linear rates of permeability with an $R^2$ value above 0.75 were used for calculating permeability. Statistical analysis of the $P_{app}$ data was compared using ANOVA, with Tukey's post analysis in GraphPad® Prism (version 6.0c, CA, USA).

### 4.3.4 Transepithelial electrical resistance

TEER was measured using an EVOM-2 TEER measurement device (Warner Instruments, Sarasota, FL, USA) attached to AgCl electrodes (Physiologic Instruments, CA, USA) immersed in 3% agar and 3M KCl. TEER measurements were taken 15, 10 and 0 min before treatment and every 5 min thereafter for the initial 20 min. Following this, TEER was measured every 20 min for an additional 2 h. Statistical analysis of TEER results was performed using Student's t-test compared to the control values. After the completion of the experiment, the tissue was removed from the chambers and fixed in 10% neutral buffered formalin for 48 h followed by storage in 70% ethanol in water. Tissues were mounted in paraffin blocks and stained using hematotoxilin and eosin (H&E) for morphological evaluation of epithelial tissue.[34]

## 4.4 Results

Caco-2 monolayers had FITC-dendrimer and $^{14}C$ mannitol $P_{app}$ values comparable to previously reported results.[14,15] The permeability of FITC-dendrimers was not significantly higher than the FITC control or 4kDa FITC dextran. Permeability results were not due to the free label as confirmed previously by size exclusion chromatography.[25] All permeability values were less than the positive control of Triton®-X-100 (Figure 4.1).

Regional differences in fluorescent PAMAM dendrimer transport were observed in isolated rat intestine. FITC-G4 and FITC-G3.5 $P_{app}$ values were significantly increased in jejunal mucosae compared to colonic. G3.5-FITC-

dendrimer transport in jejunal mucosae was greater than the respective free FITC controls in jejunum and colon tissue ($p<0.01$). On the other hand, jejunal transport of G4-FITC was not greater than FITC controls in jejunum. In colonic tissue, G3.5 and G4 transport was no greater than free FITC indicating that PAMAM dendrimer transport was greatly enhanced at the same concentration in jejunum. Differences in absorptive microvilli surface area between jejunal and colonic epithelium may be the cause of the different $P_{app}$ values even in windows of the same area.[35]

Unlabeled PAMAM dendrimers (G3.5 and G4) were observed to have differing effects on rat intestinal TEER. G3.5 and G4 dendrimers both reduced TEER with increasing concentration. The concentration at which TEER reduction was reduced was dependent on the charge of the dendrimer. 1 mM G3.5 dendrimers caused a significant decrease in TEER in jejunum between 10-40 min. time points, but returned to a non-statistically significant reduction at later time points ($p<0.05$). G4 dendrimers had no significant TEER reduction at concentrations of 1.0 mM in jejunum. A significant decrease in TEER at 10mM concentrations in rat jejunal tissue was observed for G3.5 and G4 dendrimers. G3.5 and G4 dendrimers at 0.1mM concentrations had no significant effects on jejunal TEER.

In colonic tissue the TEER values were significantly reduced by G3.5 (1 mM), while G4 dendrimers had no effect. At 10mM, both G4 and G3.5 dendrimers caused a significant reduction in TEER after 5 min ($p<0.05$). The TEER reduction was greater for G3.5 dendrimers than G4 (both 10mM), whereas

0.1 mM G3.5 and G4 caused no change.

$^{14}C$ mannitol basal $P_{app}$ values were consistent with previous literature reports for isolated rat intestinal epithelium.[30,32,36,37] An increasing trend in permeability was correlated with an increased unlabeled dendrimer concentration in isolated rat jejunum (Figure 4.3). This trend in mannitol permeability enhancement was similar for G4 and G3.5 dendrimers, although neither was significantly different from control ($p<0.05$). This trend is consistent with the inverse relationship of mannitol permeability and TEER values observed in prior experiments.[31]

Tissue morphology in isolated rat jejunum was only affected by unlabeled dendrimer concentrations in excess of 1 mM. In general, all tissues (including controls) in Ussing chambers displayed minor sloughing of epithelia, and edema due to the lack of lymphatic drainage (typical of isolated tissue models). Jejunal mucosae treated with G3.5 and G4 dendrimers at 0.1mM and 1.0mM concentrations showed no major structural difference from control. Ten mM G3.5 dendrimers caused a significant reduction in the tissue thickness along the crypt-villus axis and significant sloughing off of epithelial cells (Figure 4.4).

Colonic epithelial tissues were observed to have sloughing of surface cells at concentrations as low as 0.1mM for G3.5 dendrimers. G4 did not exhibit any significant sloughing of epithelial layers until 10mM concentrations were used. These data correspond with TEER and mannitol permeability, which are not affected by G4 at 0.1 and 1 mM concentrations, but are mildly affected by G3.5 at 1.0mM (Figure 4.5 and Table 4.1).

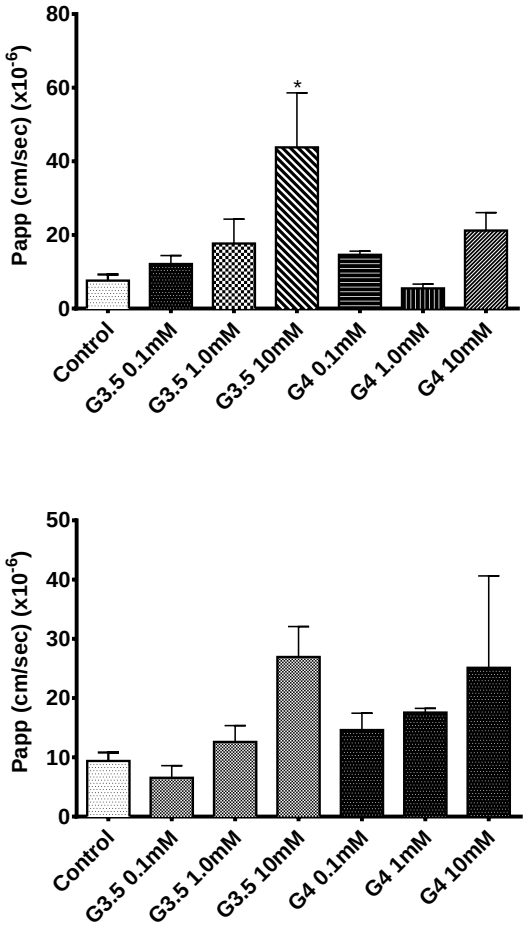


**Figure 4.3.** $^{14}C$ mannitol permeability in isolated rat intestinal tissues. Top panel: colon, bottom panel: jejunum. $^{14}C$ mannitol permeability was observed to have an increased trend in colonic epithelium when exposed to increasing concentrations of G3.5 dendrimers for 120 min. Although no concentration caused a significant increase in mannitol, the 10 mM concentrations had the greatest positive effect on mannitol permeability. G4 dendrimers had a similar effect on jejunal epithelium, with a trending increase in mannitol permeability. The effect of G4 dendrimers on mannitol permeability enhancement appeared lower in colon tissues than jejunal epithelium. The control treatment is in the presence of free FITC. No statistically significant differences occured (n=3-12).

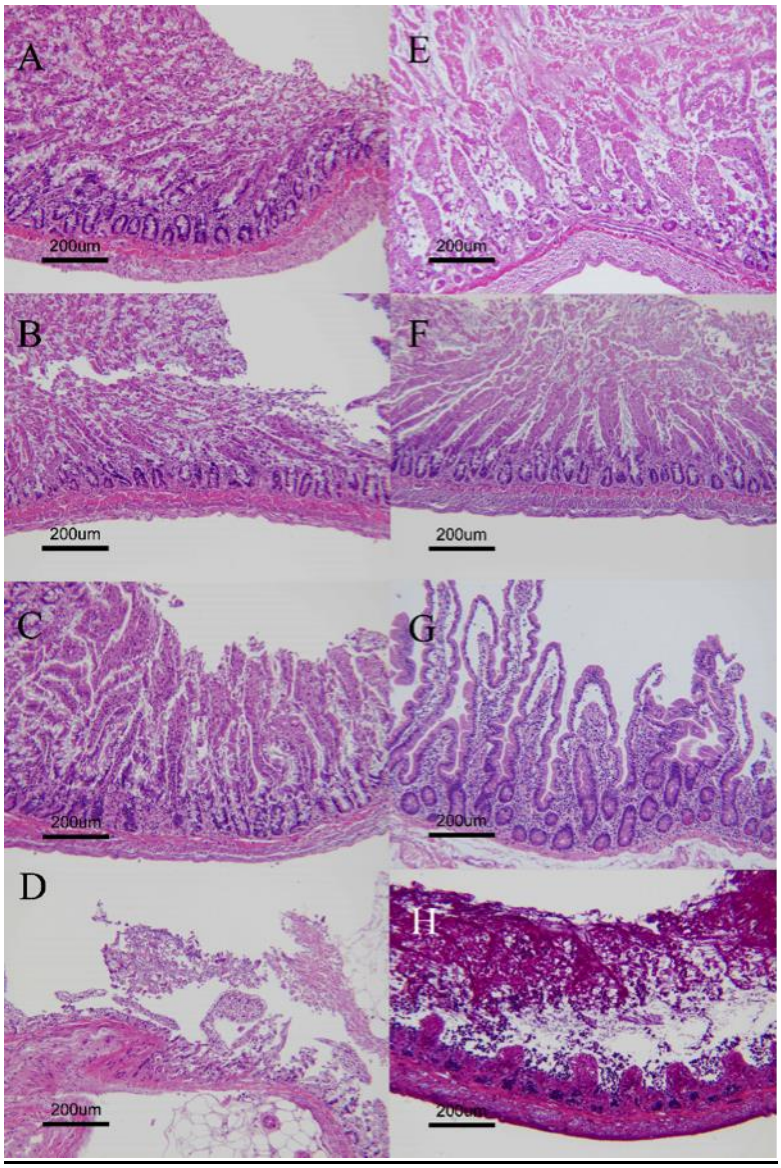


**Figure 4.4.** H&E evaluation of isolated rat jejunal epithelial tissue treated with G3.5 and G4 dendrimers for 120 min. G3.5 and G4 dendrimer treatment had no significant effect up to 1.0mM concentrations. 10mM concentrations of G3.5 and G4 caused significant reduction of tissue width along the crypt-villus axis (top to bottom) as well as major sloughing of epithelial layers. Panels are arranged in the following order: control (A, E), G3.5 0.1mM (B), G3.5 1.0mM (C), G3.5 10mM (D), G4 0.1mM (F) G4 1.0mM (G), G4 10mM (H).

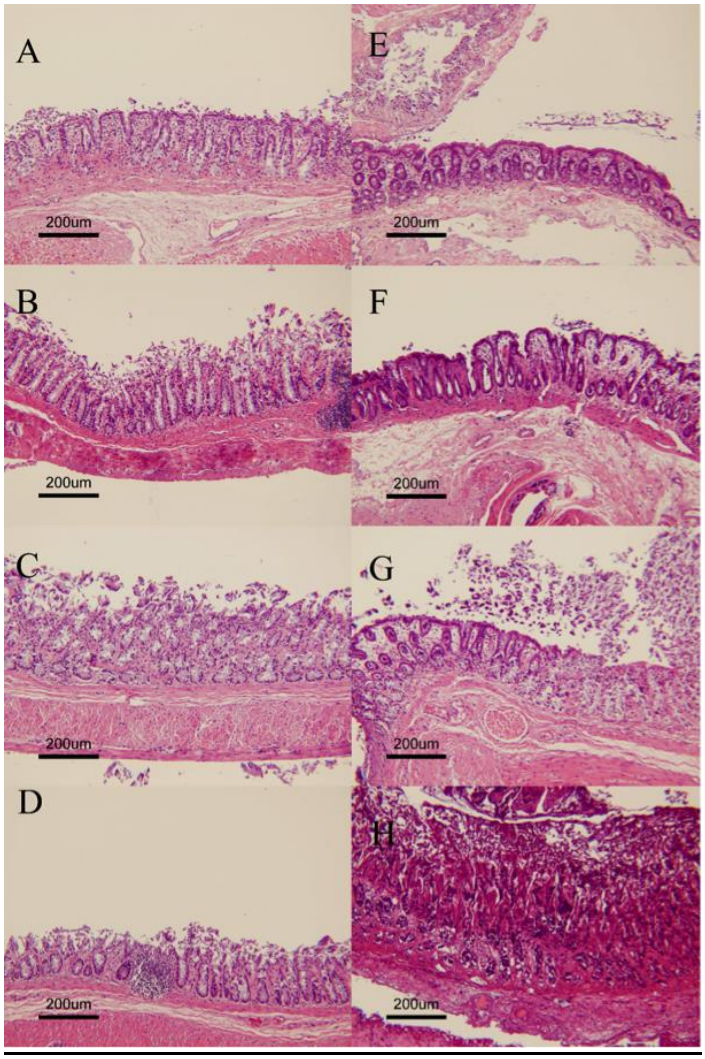


**Figure 4.5.** H&E evaluation of isolated rat colonic epithelial tissue treated with G3.5 and G4 dendrimers for 120 min. G3.5 treatments were observed to cause significant sloughing of epithelial cells at concentrations as low as 0.1mM (B, C & D). G4 dendrimers did not seem to have a serious effect on colonic morphology until 10mM concentrations were reached, where the epithelial surface was disrupted. This is consistent with TEER and mannitol data showing reduced effects in colonic epithelium. Panels are arranged in the following order: control (A, E), G3.5 0.1mM (B), G3.5 1.0mM (C), G3.5 10mM (D), G4 0.1mM (F) G4 1.0mM (G), G4 10mM (H).

**Table 4.1.** Regional dependence of FITC-PAMAM dendrimer $P_{app}$ values.

| | Rat Colonic Mucosae (x$10^{-6}$ cm/s) | | | Rat Jejunal Mucosae (x$10^{-6}$ cm/s) | | |
|---|---|---|---|---|---|---|
| | FITC | G4-FITC | G3.5-FITC | FITC | G4-FITC | G3.5- FITC |
| Mean ± Std. Dev. | 0.88 ± 0.23 | 0.43 ± 0.082 | 0.74 ± 0.42 | 2.0 ± 1.2 | *5.5 ± 2.7*[a] | *10* ± 2.8[a,b] |

a. Statistically significant difference from colon control ($p<0.05$).
b. Statistically significant difference from jejunum control ($p<0.05$).
c. Jejunal data included from previous chapter for comparison.

## 4.5 Discussion

PAMAM dendrimers have been observed to traverse the intestinal epithelium in vitro and in vivo indicating that they may be useful for oral drug delivery of associated molecules. The rate of their intestinal transport was first reported by Wiwattanapatapee et al. across everted sacs.[23] Since then, a variety of studies have shown the capacity of PAMAM dendrimers to enhance permeability.[3,8] In the studies described in this Chapter we observed that PAMAM dendrimers were capable of traversing the rat intestinal membrane to a greater extent in jejunal than colonic epithelium. This difference in transport may be due to the greater absorptive surface area of small intestine, compared to colonic epithelium, even in Ussing chambers (6/1 ratio in rat).[35] In addition, the colon of rats has a mucous layer of approximately 830 μm thick compared to the jejunum mucous thickness of approximately 123 μm.[38] Mucous layer thickness may cause differing degrees of entrapment, especially for cationic dendrimers, which are likely to interact with the anionic mucous layer and restrict access to the epithelium.[39]

TEER values for colon and jejunal epithelium treated at equal concentrations (1mM) of FITC or non-FITC G3.5 and G4 were statistically reduced only by the G3.5 treatment. This may be an effect of mucous entrapment of cationic G4 dendrimers as well. This mucus-protective effect may reduce the interaction of G4 dendrimers with epithelial layers, while anionic dendrimers penetrate the mucous layer and begin to reduce the TEER of the epithelial cells at a much lower threshold. At 10mM, both G4 and G3.5 have

similar TEER-reducing effects on colon and jejunum, likely due to the high concentration of dendrimer, which would inhibit any observable effect of mucous entrapment on TEER. Future experiments may be aimed at discovering the extent of mucus-entrapment for cationic dendrimers and the difference that charge has on this entrapment.

It appears that a similar trend is observed in colonic histology, where G3.5 dendrimers caused sloughing of epithelial layers, at much lower concentrations than G4 dendrimers. At 1.0mM exposure colon morphology was significantly affected by G3.5 treatment, whereas the epithelial surface was mostly intact for G4 treatments at the same concentration. This provides evidence that G3.5 dendrimers might cause damage to epithelial layers at low concentrations, whereas higher concentrations of G4 dendrimers are needed for such impact.

In jejunal mucosae, the tissue morphology was intact when exposed up to 1mM G3.5 and G4 dendrimer. Ten mM caused a significant decrease in TEER and a loss of epithelial layers in all tissue types and with all dendrimer generations tested. This concentration also caused a trending increase in mannitol permeability in the small and large intestinal epithelium. This is indicative of a potential upper safety limit for PAMAM dendrimer oral drug delivery. It is interesting to note that this upper limit is 10-100 fold higher in concentration than suggested by previous work in the Caco-2 model.[14,26] This result, while not surprising, confirms differences in sensitivity between isolated tissue models and cell culture.

Of significant note was the comparison of Papp values in intestinal tissue

compared to the Caco-2 models (Figure 4.6). The Caco-2 $P_{app}$ of G3.5 and G4 dendrimers was similar to that of rat jejunal mucosae. In spite of the Caco-2 cell line being derived from colonic epithelium, the permeability values observed were found to overestimate colonic epithelial transport. Permeability values obtained in Caco-2 models (especially for passively accumulated drugs) have been found to closely match jejunal transport in human tissues.[40]

While the concentration of dendrimers appears to have an impact on epithelial morphology, TEER and paracellular permeability, the actual concentration in vivo may be significantly altered by dilution, mixing, reserve time and degradation inside the gastrointestinal tract. G3.5 dendrimers appear to have greater effect on epithelial TEER and morphology at lower concentrations while G4 dendrimers appear to show observable damage to epithelial layers at higher concentrations (>10mM).

## 4.6 Conclusion

In this chapter we sought to investigate the regional dependence of PAMAM dendrimer transport. Concentrations at 10mM or above unlabeled G3.5 and G4 dendrimers incubated with isolated rat intestine for 120 min appear to negatively impact jejunal and colonic mucosal morphology, TEER and $^{14}C$ mannitol transport, indicating that these may be excessive concentrations for oral drug delivery applications in vivo. The $P_{app}$ of FITC PAMAM dendrimers in Caco-2 monolayers closely matches isolated jejunal transport. Colonic transport of FITC-PAMAM dendrimers was less than jejunal transport in isolated rat intestinal

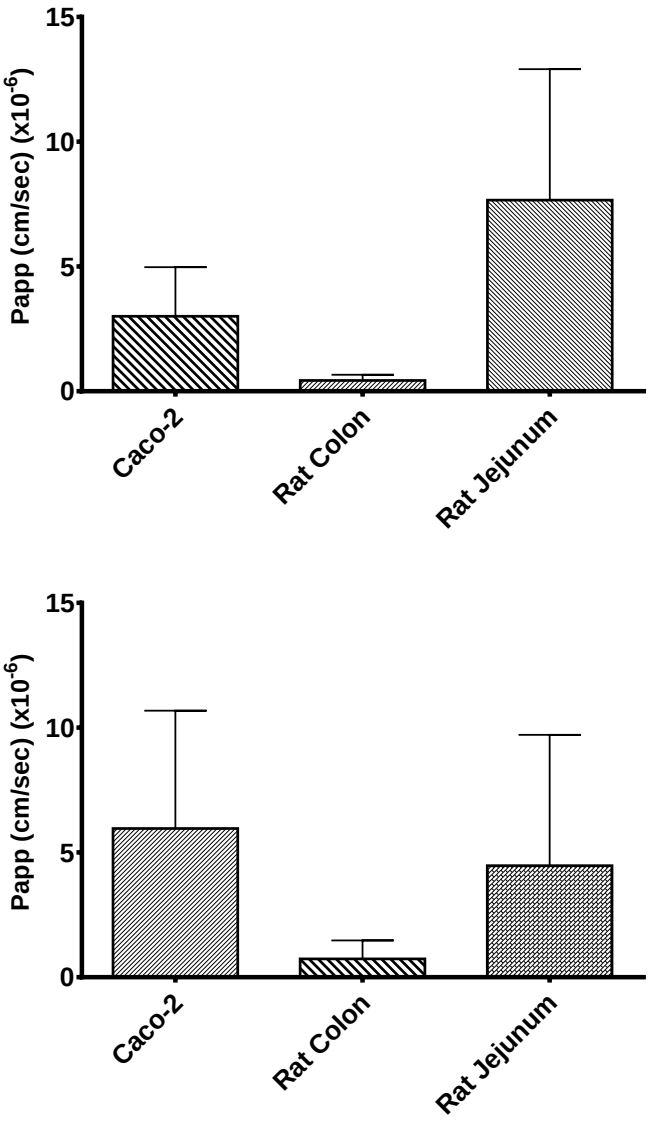


**Figure 4.6.** Caco-2 permeability compared to isolated rat tissue transport of PAMAM dendrimers. Top panel: G4 dendrimer, bottom panel: G3.5 dendrimer. The comparative transport of Caco-2 cells to isolated tissue models displays the overestimation of rat colonic transport by Caco-2 cell culture transport. Caco-2 transport of PAMAM dendrimers more closely relates to rat jejunum than rat colon. None of the groups were statistically different from the others ($p<0.05$).

epithelium. This study lays the groundwork for future work in PAMAM dendrimer oral drug delivery. The next Chapter is focused on using isolated human tissue mucosae in order to effectively estimate the human fraction absorbed of PAMAM dendrimers.

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# CHAPTER 5

# TRANSEPITHELIAL TRANSPORT OF PAMAM DENDRIMERS ACROSS ISOLATED HUMAN INTESTINAL TISSUE

## 5.1 Introduction

PAMAM dendrimers are a class of polymeric nanoparticles that have shown potential in drug delivery (Figure 5.1).[1–3] These constructs have a hyper-branched structure that allows for the attachment of multiple therapeutic and imaging moieties to surface groups.[4] They are synthesized with repeating alternating units of ethylene diamine and methyl methacrylate.[5] The surface charge of dendrimers can be controlled based on functionalization of dendrimer termini with carboxyl, amine or hydroxyl groups. The hydrophilic structure of dendrimers can be utilized to enhance the solubility and intestinal permeability of drugs covalently attached or admixed with dendrimers.[6,7] These properties provide rationale for the application of PAMAM dendrimers in drug delivery.

Given their macromolecular nature, appreciable transport across intestinal epithelial barrier has been observed providing rationale for the development of PAMAM dendrimers as oral drug carriers.[3,8] Dosing of drugs via the oral route has many advantages over parenteral administration including patient

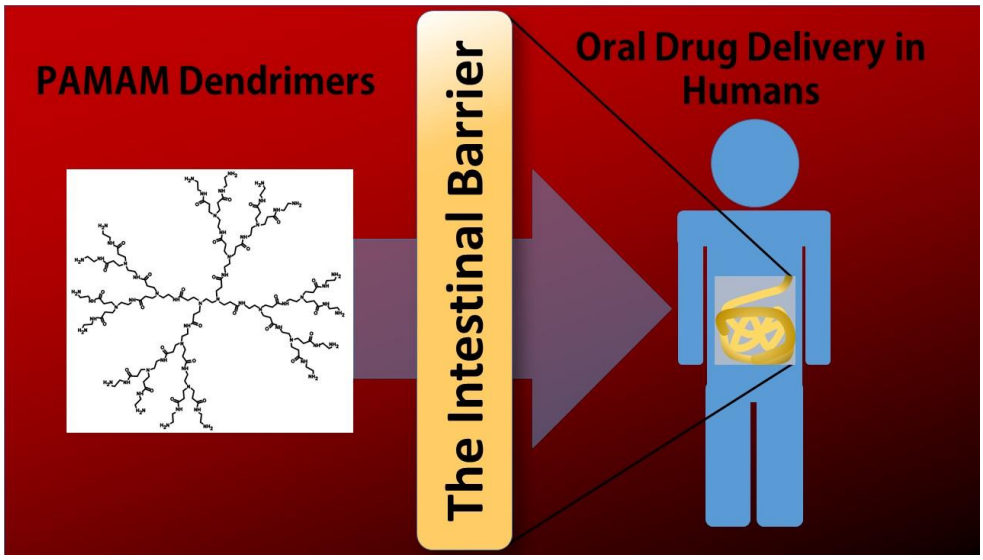


**Figure 5.1.** Challenges to dendrimer oral drug delivery lie in the transepithelial transport across human intestinal barriers.

preference, cost-effectiveness and enhanced patient quality of life.[9] PAMAM dendrimers have the ability to enhance the oral permeability of drugs based on previous work in Caco-2 cell culture, isolated tissue and in vivo models.[1–3,6,8,10–12]

Dendrimer transport in Caco-2 monolayers has been observed to be size, charge, concentration and incubation-time dependent.[10,11] The transepithelial transport of dendrimers ranging from generation 1 (G1) to generation 4.5 (G4.5) has been observed in Caco-2 studies. The apparent permeability ($P_{app}$) was observed to vary with increasing generational size in anionic G1.5-3.5 dendrimers.[10,13] Rates of transport were also found to increase with increasing incubation time for positively charged G0 and G1 dendrimers in Caco-2 monolayers.[10] The $P_{app}$ of G3.5-FITC labeled dendrimers was found to be ~$6\times10^{-6}$ cm/s at 120 min incubation times and 1.0mM concentrations in Caco-2 cells.[13] Concentrations of anionic G3.5–COOH terminated dendrimers greater than 1.0mM were found to be toxic via lactose dehydrogenase (LDH) release in Caco-2 cells at 150 min incubation times.[14] Cationic G4-$NH_2$ dendrimers were found to be toxic at concentrations of 0.1mM via LDH release, unless surface groups were substituted with neutral of hydrophobic moleceules.[10,15] Kitchens et al. noted that 1.0mM G4-FITC dendrimers with a 1:8 loading ratio and 120 min incubation time did not induce cytotoxicity via the WST-1 assay. The $P_{app}$ of this compound was ~$24\times10^{-6}$ cm/s.[13]

Work in everted rat intestinal tissue models has found that the endocytic index of PAMAM dendrimers was higher for anionic G5.5 PAMAM dendrimers than cationic G4 dendrimers at similar concentrations and incubation times.[3]

G3.5-FITC labeled dendrimers were found to have a permeability higher than that of control and G4-FITC dendrimers at 1.0mM concentrations in isolated rat jejunum mounted in Ussing chambers.[2] Further work in isolated tissue models using rat jejunum has shown that 1.0mM concentrations of G3.5 and G4 dendrimers caused no significant damage to isolated tissue compared to controls. Discrepancies in the toxic concentration of G4 dendrimers between Caco-2 cells and isolated rat intestinal models may be due to the lack of mucous, morphology and other factors present in isolated tissue models.

Further research *in vivo* has shown that PAMAM dendrimers are able to cross the intestinal epithelium and enter the systemic blood circulation. Thiagarajan et al. observed an oral fraction absorbed of 9.4% in CD-1 mice dosed with G6.5 anionic dendrimers.[1] Evidence also exists that PAMAM dendrimers have the ability to enhance the permeability of drugs mixed with or encapsulated in dendrimers. Sadekar et al. noted the ability of PAMAM dendrimers to increase the oral area under the curve (AUC) of camptothecin two fold when dendrimers were dosed at 1000mg/kg (~7mM intestinal concentration) to CD-1 mice.[6] Additional studies on the intestinal morphology after said treatment noted a lack of histopathological changes in the villi structure.

While these cell culture and animal studies have established the groundwork for PAMAM dendrimer oral delivery, translation into clinical use has not yet been accomplished. Significant differences exist between human, rat and Caco-2 permeability studies including transport proteins, metabolic proteins, morphological structure and lymphoid tissue.[16–23] Our aim in this study is to

predict the human intestinal permeability of PAMAM dendrimers based on isolated human tissue results.[24] This information will provide predictions of the applicability of PAMAM dendrimer drug delivery systems (or lack thereof) based on the permeability values obtained from human isolated tissue as well as a basic understanding of the macromolecular relationship between Caco-2, rat and human permeability experiments using PAMAM dendrimers as a test probe.

## 5.2 Materials

Fluorescein isothiocyanate (FITC), fluorescein isothiocyanate dextrans**,** N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) were obtained from Sigma Aldrich (MO, USA). $^{14}$C mannitol (0.1mCi/mL in sterile water) was obtained from American Radiolabeled Chemicals (MO, USA). PAMAM dendrimers (G3.5 and G4.0) were obtained from Dendritech, Inc. (MI, USA). All other reagents were obtained from VWR.

## 5.3 Methods

FITC labeled dendrimers (G3.5 and G4) were synthesized as reported previously.[13] PAMAM dendrimers that had carboxyl termini were first modified with ethylenediamine to create sites for FITC attachment. G3.5 dendrimers were reacted with EDC and ethylenediamine in PBS pH 7.4 followed by overnight stirring and then addition of FITC (<5mg/mL in acetone) to the amine modified dendrimer. G4 dendrimers were mixed with FITC in acetone and PBS. All solutions were then dialyzed continuously against deionized water and purified

by size exclusion chromatography (SEC) using a fast protein liquid chromatography (FPLC) system (GE Lifesciences, NJ, USA) on a preparative XK 26/70 column packed with Superdex 30 media. Final characterization of the fluorescent compounds was carried out using SEC on FPLC with a Superose 6 column (GE Lifesciences, NJ, USA) with PBS as an eluent to verify the absence of free label.

Human tissue use was approved by the University of Utah Institutional Review Board (IRB protocol #10924). Isolated jejunum and colon segments were received from colectomy, pancreaticoduodenectomy and Roux-en-Y gastric bypass surgery patients with informed consent from each patient. The patient population age ranged from 19 to 87 years of age with an average age of 58 (Table 5.1).

The majority of patients donating colon tissues were undergoing treatment for cancer. Bariatric surgery was the primary source of jejunal epithelium and thus it came from non-cancer bearing patients. Samples were in general cancer-free and non-inflamed. Any necrotic, inflamed or cancerous tissue was removed by the hospital pathology department prior to disbursement for use. One tissue segment was confirmed by pathology to contain cancerous tissue and was subsequently excluded from the study.

Healthy tissue samples obtained from surgeries were immediately immersed in ice cold oxygenated Krebs-Hensleit (KH) buffer at pH 7.4 after disbursement and transported to the lab for mounting. All further dissection was performed in a custom designed oxygenated tissue dissection bath with constant

**Table 5.1.** Patient statistics and transport time of the intestinal samples used in the study.

| | Age (yrs) | N= | Gender Ratio (M/F) | Time to Lab (H:MM) |
|---|---|---|---|---|
| Jejunum | 47 ± 13 | 11 | 0.57 | 0:19 ± 0:06 |
| Ascending Colon | 65 ± 19 | 4 | 0.67 | 0:16 ± 0:02 |
| Transverse Colon | 62 ± 15 | 14 | 0.56 | 0:28 ± 0:14 |
| Sigmoid Colon | 55 ± 16 | 6 | 1.67 | 0:23 ± 0:08 |
| Males | 55 ± 18 | | - | 0:21 ± 0:07 |
| Females | 60 ± 14 | | - | 0:24 ±0:14 |
| Total | 58 ± 16 | 35 | 0.83 | 0:22 ± 0:10 |

bubbling of 95% $O_2$/5% $C0_2$. The external muscularis layer was dissected away and the remaining epithelium was mounted in Ussing chambers with P2304 inserts (Physiolgic Instruments, CA, USA) and an exposed surface area of 0.3 $cm^2$. Chambers were filled with 5mL of 37°C Krebs-Hensleit buffer gassed with 95% $O_2$/5% $CO_2$ and equilibrated for 45 min prior to application of treatment. At time t=0min solutions containing test probes were added to the apical side of the chambers. Unlabeled dendrimers were added at concentrations of 0.1, 1.0, or 10mM. All fluorescently labeled dendrimers were added at a concentration of 1.0mM. Triton X100 was added at a 10% v/v concentration. All experiments included 5µL of $^{14}$C-mannitol. The apical and basolateral side of the chambers were sampled (200µL) at t=0 min and volume was replaced with KH buffer warmed to 37°C and oxygenated to maintain constant volume. The apical side was sampled at t=0 and 120 min while the basolateral side was sampled at t=0 min followed by 20 min intervals for 120 min. Samples were analyzed using spectrophotometry (Spectramax M2, Molecular devices, CA, USA) and liquid scintillation (LS-6000IC, Beckman, CA, USA).

TEER was measured using Evom-2 TEER measurement device (Warner Instruments, FL, USA) attached to AgCl electrodes (Physiologic Instruments, CA, USA) that were previously filled with a 3% agar solution made in 3 M KCl. TEER measurements were taken 15, 10 and 0 min before treatment and every 5 min after treatment began for the initial 20 min. Following the initial 20 min, TEER was measured every 20 min for 2 h. TEER results were analyzed using a student's t-test to compare treatment values to the control.

At the end of the experiment the tissue was carefully removed from the chambers and fixed in buffered formalin. Tissues were mounted in paraffin blocks and stained using hematotoxilin and eosin (H&E).[25]

Statistical analysis of the $P_{app}$ data was compared using ANOVA, with Tukey's post analysis in GraphPad® Prism (version 6.0c, CA, USA). TEER values were compared to control using one-way student's t-test.

## 5.4 Results

Human tissue samples were obtained from the campus tissue repository approximately 50 min after removal from the patient. Samples were obtained and then transported to the lab in the times listed in Table 5.1. Control mannitol permeability and TEER values obtained for jejunum and colon tissue were within expected ranges based on previous literature.[24] Tissue maintained morphological structure as noted by histological evaluation of the epithelial barriers. Mild edema was noted throughout the tissue specimens retrieved from Ussing chambers which is likely a result of the removal of lymphatic drainage in isolated tissue models. Overall the tissue morphology and integrity was intact as evidenced by mannitol, TEER and histological evaluation and in accordance with previous literature on similar experimental methods (Figures 5.2, 5.3, 5.4 and 5.5).[26]

The permeability of PAMAM dendrimers was evaluated in this study along with the penetration enhancing effects on the small paracellular marker mannitol. G3.5 and G4 PAMAM dendrimers did not cause a statistically significant increase in mannitol transport at 0.1mM and 1.0mM concentrations across human jejunum

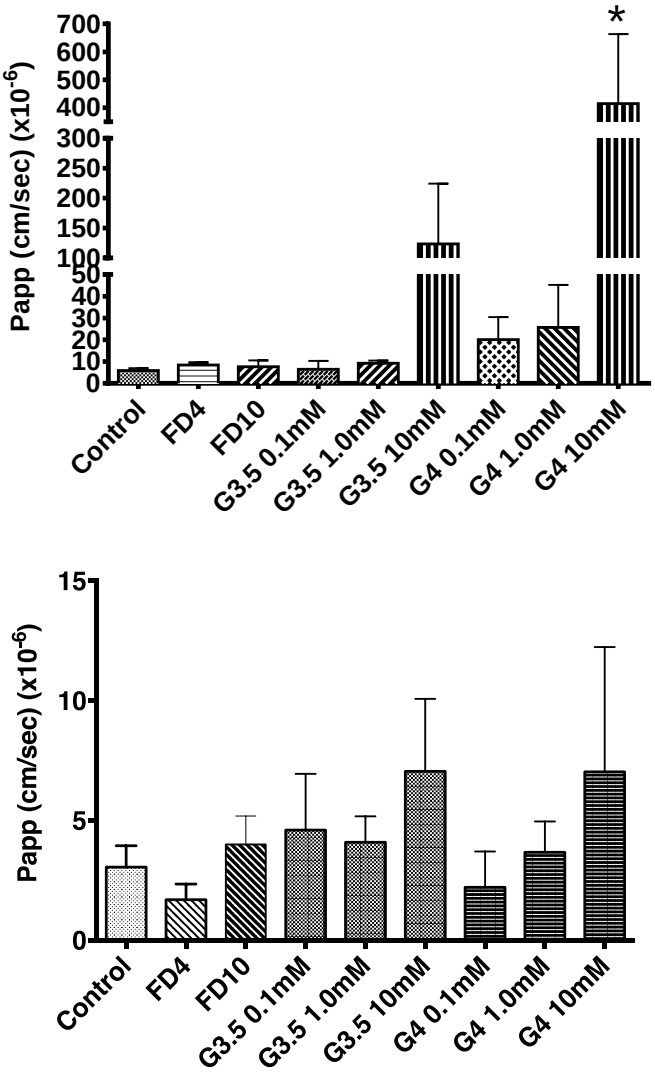


**Figure 5.2.** PAMAM dendrimer mannitol permeability in human intestinal epithelium; top panel: colon, bottom panel: jejunum. The permeability of mannitol was significantly increased compared to control by G4 10mM treatments in human colon ($p<0.05$). Mannitol permeability in human jejunum trended toward increased permeability at higher concentrations of dendrimers, but this increase was not statistically significant (n=3-19); * signifies statistically significant from control, FD4=4kDa FITC dextran, FD10=10kDa FITC dextran, mean ± standard error of the mean).

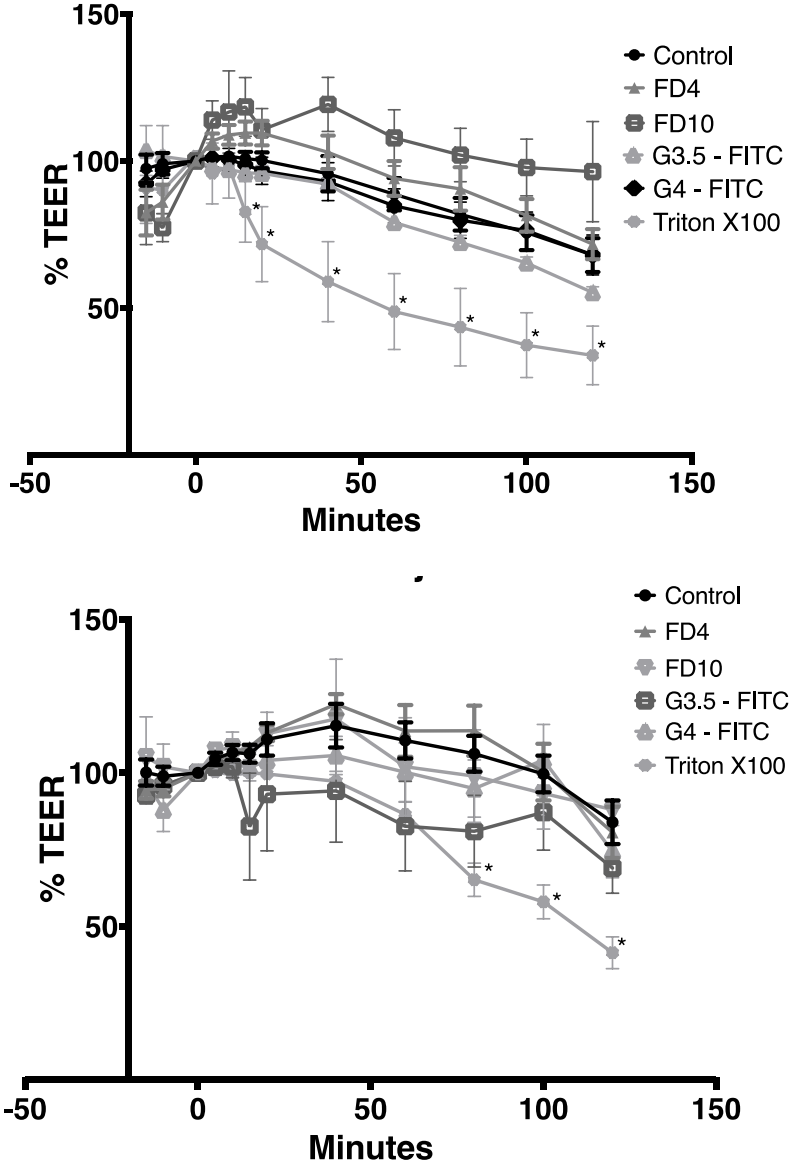


**Figure 5.3.** Percent TEER values for human colonic and jejunal mucosae. Top panel: colon, Bottom panel: jejunum; (mean ± standard error of mean, * signifies $p<0.05$ compared to control). FD4=4kDa FITC dextran, FD10=10kDa FITC dextran.

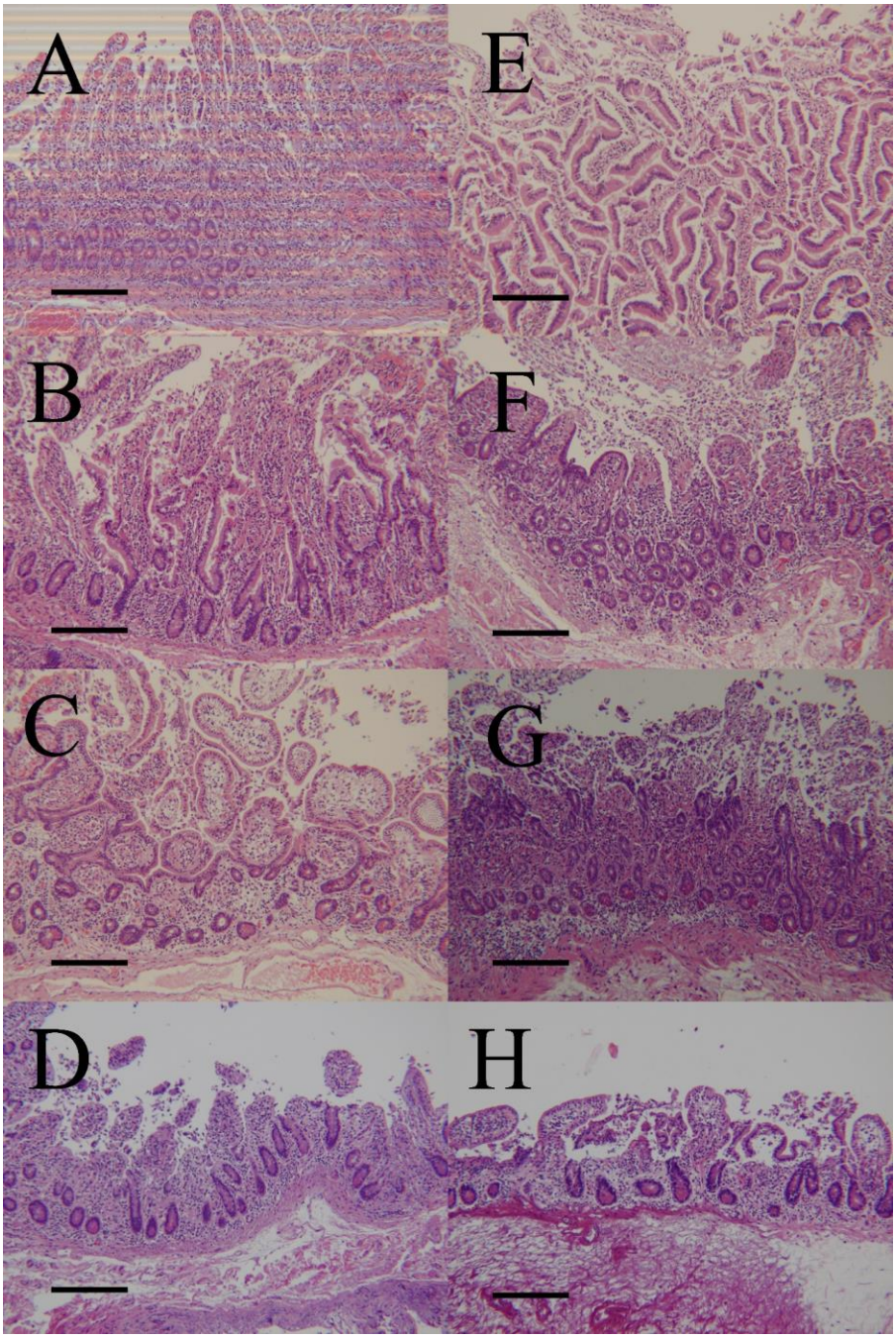


**Figure 5.4.** Human jejunum histology in the presence of 0.1mM, 1mM and 10mM PAMAM dendrimers. The histological evaluation of treatment of human tissue with PAMAM dendrimers showed significant reduction in the crypt-villous axis tissue thickness when treated with 10mM concentrations of G3.5-COOH and G4-$NH_2$ dendrimers. G3.5-COOH and G4-$NH_2$ dendrimers at 0.1mM and 1.0mM concentrations did not cause a significantly observable reduction in thickness of the epithelium along the crypt-villous axis (top to bottom). ((A), (E) controls; (B) 0.1mM G3.5; (C) 1mM G3.5; (D) 10mM G3.5; (F)1mM G4; (G) 1mM G4; (H) 10mM G4).

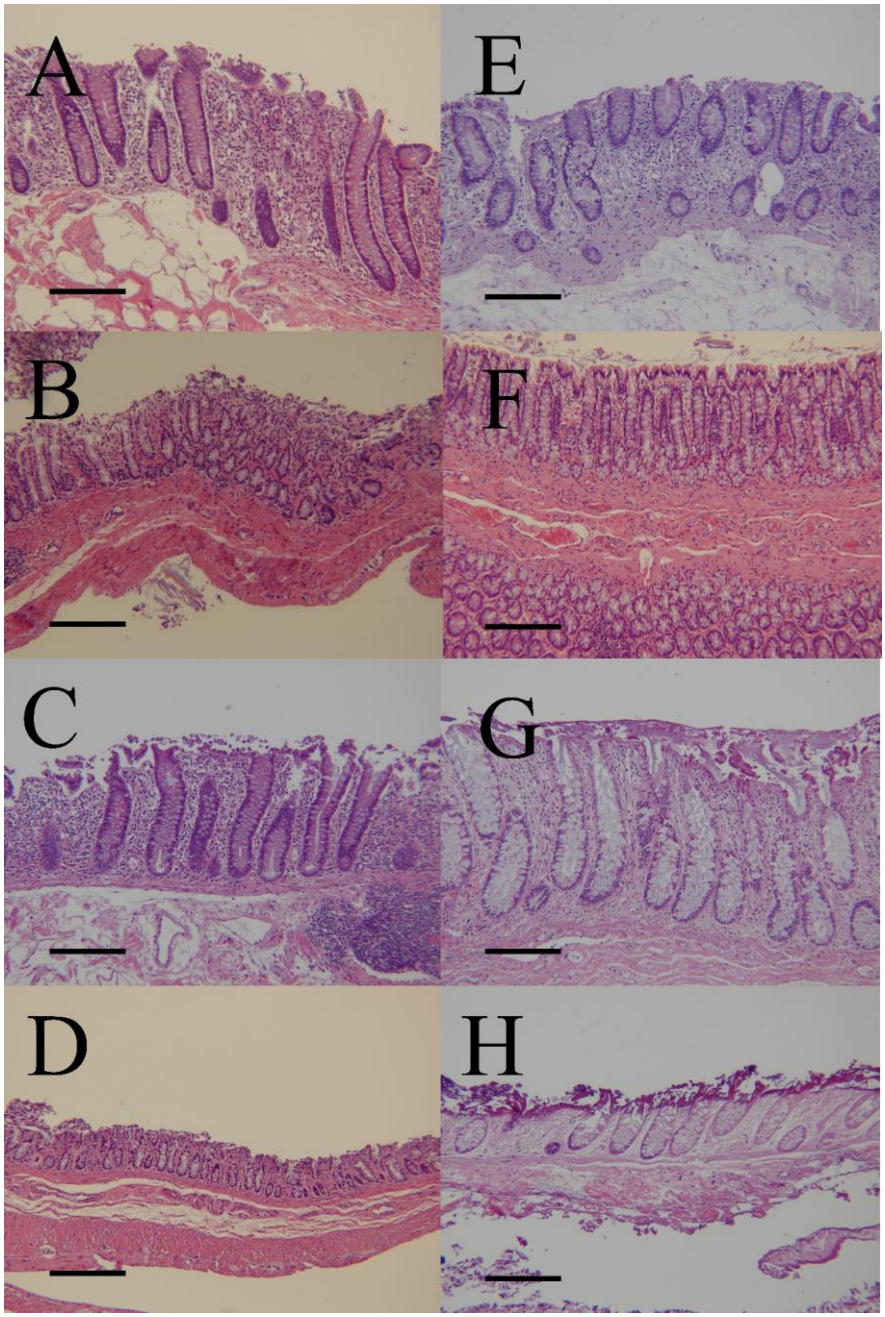


**Figure 5.5.** Human colon histology in the presence of 0.1mM, 1mM and 10mM PAMAM dendrimers. Colonic epithelium was similarly affected by PAMAM dendrimers as jejunal isolated tissues. The tissue thickness was reduced with high concentrations of PAMAM dendrimers (D, H). Minor sloughing of epithelial cells and edema was noted in all samples including controls. This sloughing of epithelial cells from the surface was significantly greater in tissues treated with 10mM concentrations of dendrimers. ((A), (E) controls; (B) 0.1mM G3.5; (C) 1mM G3.5; (D) 10mM G3.5; (F)1mM G4; (G) 1mM G4; (H) 10mM G4).

and colonic tissues. Ten mM concentration of G4 dendrimers caused a significant increase in mannitol permeability compared to control in human colon tissue, but not in human jejunum (Figure 5.2).

FITC-labeled PAMAM dendrimer permeability was not statistically different from free FITC in jejunum and colonic epithelium. G3.5 and G4 FITC labeled dendrimers (1.0mM) trended above the control in colonic segments, but did not attain a statistically different value from control. The permeability values for G3.5-COOH and G4-$NH_2$ dendrimers in human colon was $3.65 \times 10^{-6}$ cm/s and $2.08 \times 10^{-6}$ cm/s, respectively. The $P_{app}$ for G3.5-COOH and G4-COOH dendrimers in jejunal tissue was $2.11 \times 10^{-6}$ cm/s and $0.96 \times 10^{-6}$ cm/s, respectively (Figure 5.6).

TEER values at t=0 min were an average of 54±25 ohms-$cm^2$ and 150±73 ohms-$cm^2$ for colon and jejunum, respectively. TEER values in colonic and jejunal mucosae did not differ from the control for G3.5 and G4 PAMAM dendrimers at 1.0mM concentrations. This is similar to previous data obtained in rat epithelium.[2] A reduction of 50% of the initial TEER value was not achieved for any samples excluding the positive control Triton X100.

Histological evaluation of the tissue noted the apparent sloughing of cells off the epithelial surface at 10mM concentrations of G3.5 and G4 dendrimers. Both of these probes caused significant reduction in the epithelial thickness at 10mM concentrations compared to 0.1 or 1mM concentrations and control. The apparent change in epithelial thickness along the crypt to villous axis is likely due to the negative interaction of 10mM concentrations of dendrimers with epithelial cell layers. The colonic and jejunal tissues showed a similar trend in surface

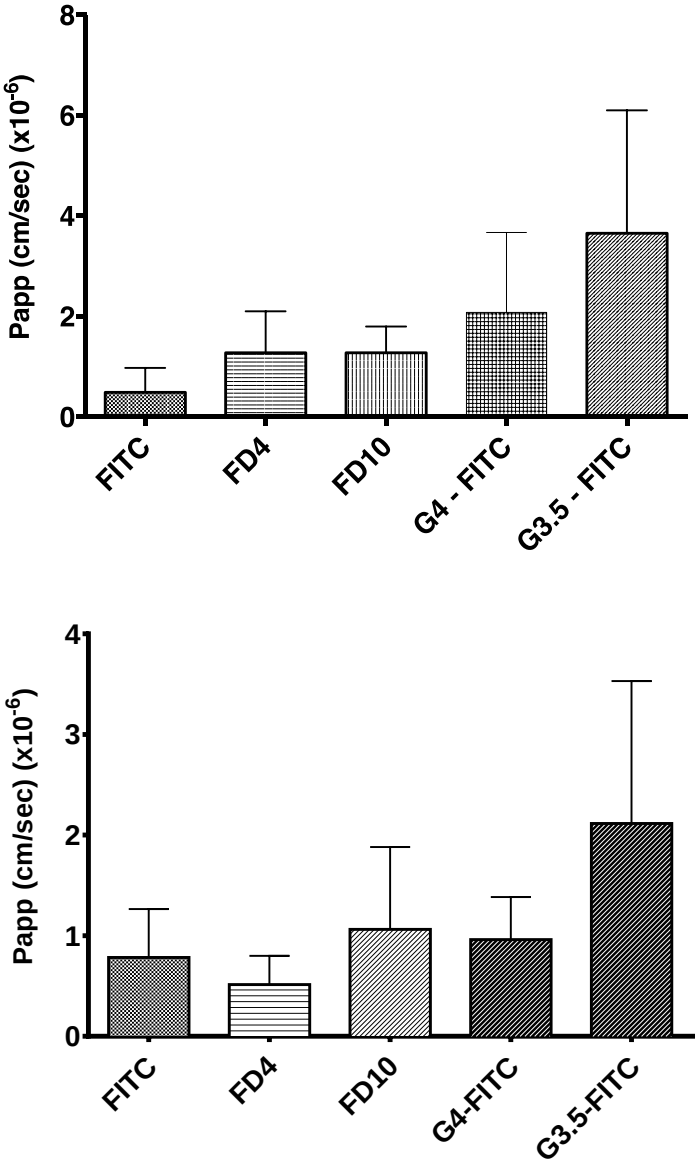


**Figure 5.6.** Apparent permeability of PAMAM dendrimers across isolated human intestinal tissue; top panel: colon, bottom panel: jejunum. G3.5-FITC was observed to have the highest $P_{app}$ of the groups tested in colonic and jejunal segments. No significant differences were observed. (n=3-6, FD4=4kDa FITC dextran, FD10=10kDa FITC dextran, mean ± standard error of the mean).

epithelium disruption when treated with 10mM concentrations of G3.5-COOH and G4-$NH_2$ dendrimers. The deeper layers of epithelium closer to the lamina propria remained intact even at 10mM concentrations of dendrimer (Figure 5.5, 5.6).

## 5.5 Discussion

PAMAM dendrimers' oral delivery is highly dependent on their permeability across the intestinal epithelium. The intestinal barrier blocks hydrophilic macromolecules from penetrating the epithelium and entering systemic circulation.[27–31] Substantial absorption across the gastrointestinal tract in rat models was observed suggesting the potential for improving the delivery of highly potent drugs with limited oral bioavailability. These studies were done in cell culture and animal models, which lack many aspects of human intestinal epithelium.[16,19,20,32] The next logical step was to test their permeability across human tissue. In this study we used isolated human intestinal tissue to evaluate PAMAM dendrimer transepithelial transport.

Consistent with previous reports, only minor changes were observed in intestinal morphology from fresh tissue and tissue after being treated in Ussing chambers for the experimental time frame of 120 min.[33,34] Slight edema appeared in all sections treated in Ussing chambers that corresponded with the lack of lymphatic drainage in this set up, but this did not inhibit the viability of the tissue as previously observed.[26]

Significant differences were noted between previous Caco-2 and isolated human intestinal TEER. Human TEER values were not significantly reduced for

any treatment group compared to control, except the positive control Triton X100. Previously we noted that concentrations as low as 0.1mM of G4 PAMAM dendrimers caused a reduction in TEER in Caco-2 monolayers. This concentration is 10 fold lower than concentrations used in this study.[10,14] Caco-2 cells are thus noticeably more sensitive to reductions in TEER than isolated tissue models. These results are confirmed by previous isolated rat intestinal tissue results using 1mM concentrations of G3.5 and G4 dendrimers.[2]

The discrepancy between Caco-2 cells and isolated tissue extends to their morphology. Previously we had shown that positively charged G4-PAMAM dendrimers cause significant damage to Caco-2 microvilli at 1mM concentrations for 120 min incubation times.[35] In human isolated tissue no such morphological changes occurred at similar concentrations and incubation times for G3.5-COOH and G4-$NH_2$ dendrimers. This difference between the maximum tolerated concentrations of dendrimers rationalizes the use of isolated tissue models for understanding epithelial toxicity and transport. The difference between Caco-2 and isolated tissue models may be caused by the lack of mucous, supportive cells and morphology which are present in isolated tissue models.[17,36,37]

The permeability of mannitol was significantly increased in colonic epithelium treated with G3.5-COOH and G4-$NH_2$ dendrimers at 10mM concentrations. 10mM concentrations did not however significantly enhance the mannitol permeability in jejunal segments. Colonic epithelium have been noted for the increased sensitivity to penetration enhancement and this may be the rationale for these results in the presence of PAMAM dendrimers.[38] Factors that

may contribute to this enhanced sensitivity may be the heightened ability of colonic tissue to absorb water and electrolytes which may drag dendrimers into close proximity with epithelial layers and modulate tight junction opening and enhanced mannitol transport. One mM and 0.1mM concentrations of PAMAM dendrimer caused no significant changes in mannitol transport in colonic epithelium.

Previous studies have noted the toxic effects of PAMAM dendrimers to epithelial barriers and in vivo organ systems.[39–41] In vivo studies aimed at establishing the oral maximum tolerated dose (MTD) of PAMAM dendrimers found that G3.5 and G4 dendrimers could be given at 300 mg/kg to CD-1 mice with no adverse events.[42] A 300 mg/kg dose of G4-$NH_2$ and G3.5-COOH dendrimers is roughly equal to 1-2mM concentration at the site of the small intestine (estimated 2 fold intestinal dilution factor and 200µL gavage volume). Thus previous MTD studies agree with results here where there are no significant changes in intestinal morphology, TEER and mannitol $P_{app}$ at 1mM concentrations of G4-$NH_2$ and G3.5-COOH. Interestingly, doses of 1000mg/kg of G3.5 dendrimer (~4-7 mM epithelial concentration) given to CD-1 mice have also been observed to cause no morphological changes to epithelial barriers and no increase in mannitol absorption after 4 h treatment.[6] The signs of intestinal morphology damage and increased mannitol permeability at 10mM concentrations of G3.5 dendrimers imply an oral MTD for G3.5 dendrimers between 1000-3000 mg/kg. This is based on extrapolation of the 10mM concentrations used in Ussing chambers to animal doses and has limitations

based on variations in residence time in the gastrointestinal tract and dilution factors which are not represented in the Ussing chamber model. Nonetheless this MTD would not be surprising, since the lethal dose 50 ($LD_{50}$) of chitosan and polyacrylic acid is 1.5g/kg and 2.5g/kg, respectively.[43,44]

The results of this study conclude that concentrations of 10mM dendrimer, regardless of surface charge or region, caused changes in epithelial morphology and TEER in isolated human intestinal epithelium. Ten mM concentrations of G3.5 and G4 dendrimers may cause damage to the surface epithelial layers due to the pore-forming nature of PAMAM dendrimers within epithelial layers as investigated by molecular dynamic simulation.[45]

Previous data from our lab have established the $P_{app}$ in rat and Caco-2 cell cultures.[2] Small and large molecular weight compounds appeared to have distinct model dependent trends (Figure 5.7). Rat and human FITC $P_{app}$ closely corresponded. FITC $P_{app}$ was overestimated by Caco-2 monolayers compared to human intestinal $P_{app}$. While the wide variability of Caco-2 studies has been noted in the literature, results points out an overestimation of $P_{app}$ for small molecular weight drugs compared to human intestinal transport.[46]

The transepithelial transport of 4kDa dextran in human jejunum and colon was similar to Caco-2 results. However, G3.5 and G4-FITC labeled dendrimers' $P_{app}$ was overestimated by both rat jejunum and Caco-2 models. This trend may be due to the lack of mucous in Caco-2 monolayers which may interact with dendrimers and inhibit their transport. Such mucous interaction may be insignificant to dextran transport.

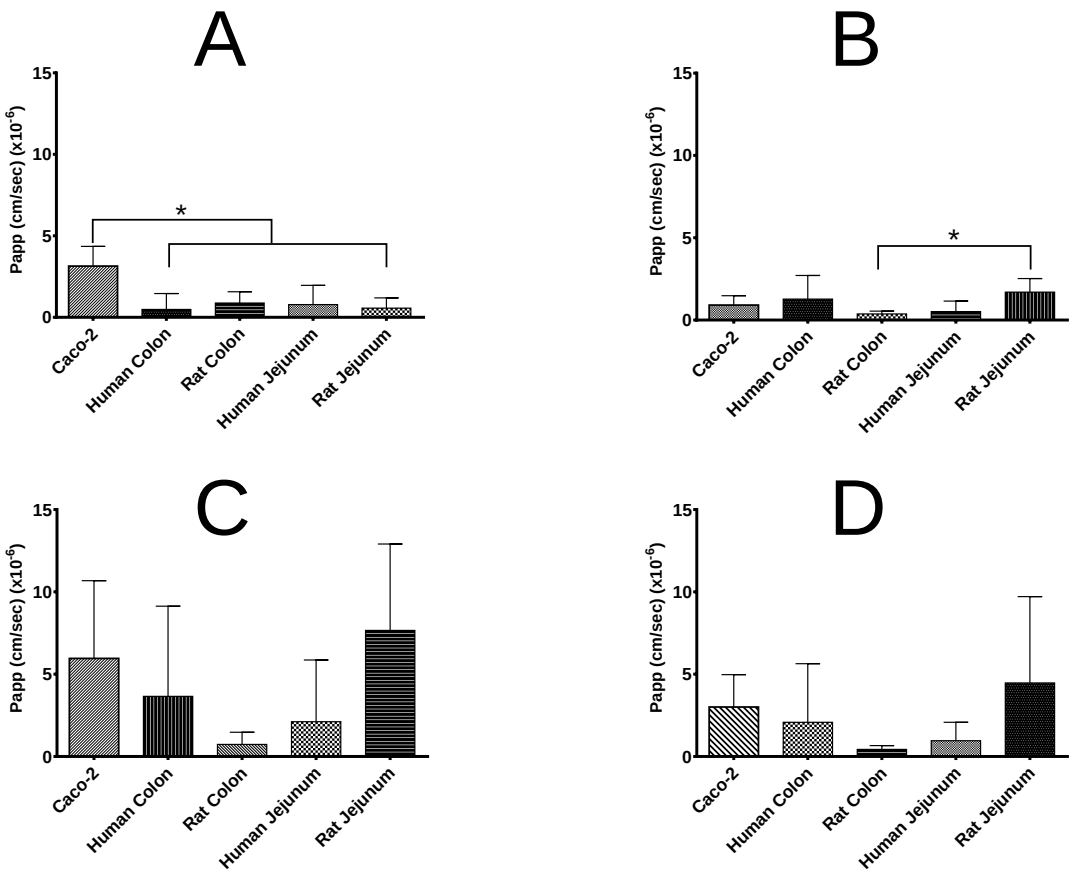


**Figure 5.7.** Comparison of Caco-2, and isolated rat and human intestinal permeability (A – FITC, B – 4kDa FITC dextran, C – G3.5-FITC, D – G4-FITC). $P_{app}$ of the respective marker in human and rat jejunum and colon, and Caco-2 models was compared. Caco-2 and rat jejunum $P_{app}$ tend to overestimate human $P_{app}$. Rat jejunal permeability from Ref. 2. Rat colonic and Caco-2 data are unpublished data. (* represent $p<0.05$ between groups divided at by star, Papp is mean ± standard error of the mean)

The overestimation of dendrimer transport by isolated rat jejunum may be due to the wide difference in number of follicles per $cm^2$ of intestinal tissue (0.03 follicles/$cm^2$ versus 2.1 follicles/$cm^2$ in human and rat small intestine, respectively).[22,47] This may represent an interesting trend in dendrimer transepithelial transport, as enhanced transport of nanoparticles through Peyers patch tissue has previously been noted and may have an effect on dendrimer transport as well.[48] Further experimentation in this area is warranted. Other differences may be due to the large variation between rat and human cell surface protein expression, and effective surface area.[49]

Isolated human tissues in Ussing chambers have been observed to have a strong correlation to human fraction absorbed.[24] For this reason we have evaluated PAMAM dendrimer permeability across isolated human tissue. The permeability of PAMAM dendrimers across isolated tissue reveals important aspects of their transepithelial transport. In the present study the permeability of G3.5 and G4 PAMAM dendrimers in human isolated jejunum was found to be $2.1\times10^{-6}$ cm/sec and $0.96\times10^{-6}$ cm/sec for G3.5 and G4 dendrimers, respectively. The colonic transport was found to be $3.65\times10^{-6}$ cm/sec and $2.08\times10^{-6}$ cm/sec for G3.5 and G4 dendrimers, respectively. If one were to estimate the fraction absorbed based on previous Lennernäs multilaboratory drug correlation curves established in human isolated tissue, the $P_{app}$ would predict a fraction absorbed of roughly 30-45% in human jejunum and 10-15% in human colon.[24]

The Lennernäs multilaboratory drug correlation study was a 12-year study monitoring the isolated human intestinal transport of >60 drugs in over 159

donors across 3 different labs. This landmark study established a strong correlation between human intestinal permeability and human fraction absorbed. The rate of permeability though the intestinal tract is a direct measure of the amount of drug penetrating through the epithelium (i.e., entering systemic circulation) and thus the correlation is highly accurate. The value of permeability in human jejunum attained in this study was very high considering the macromolecular nature of PAMAM dendrimers. It was also high in rat intestinal tissue as shown in Chapter 2 and 3, and previous studies in other labs.[3] Other proteins (ovalbumin, α-lactalbumin), and polymers (4.4kDa Dextran, 70 kDa dextran, 4 kDa PEG) have a $P_{app}$ ranging from $0.01\text{-}0.1\times10^{-6}$ cm/s.[50] Observed fraction absorbed for these macromolecules is exactly as predicted by the Lennernäs paper.[29,51,52] This provides evidence that the correlation remains valid for macromolecules.

The Lennernäs correlation curves are made from $P_{app}$ of small molecular weight pharmaceuticals and have limitations when extrapolated to PAMAM dendrimers. The prediction of fraction absorbed using small molecular weight probes across the intestinal epithelial barriers has inherent assumptions that these probes are not influenced by endocytic mechanism such as which affect PAMAM dendrimer transport. Our previous in vitro studies showed that PAMAM dendrimers are endocytosed and thus may have exceptional properties, which may limit the prediction of fraction absorbed. In addition PAMAM dendrimers are known to have penetration enhancing effects and open the tight junctions. As has often been said, the only model for man is man.[16]

This predicted fraction absorbed is based on correlation curves made from $P_{app}$ of small molecular weight pharmaceuticals and has limitations when extrapolated to macromolecules such as PAMAM dendrimers. It must be noted that the prediction of fraction absorbed using small molecular weight probes across the intestinal epithelial barriers has inherent assumptions that these probes do not influence the transport properties of epithelial barriers, whereas from our previous in vitro studies it is known that PAMAM dendrimers interact with the epithelial barriers, modulate tight junctions and are transported by a combination of paracellular and transcellular route.[10] It has already been observed that dendrimers are endocytosed into the intracellular compartment, by Caco-2 studies.[53] This could lead to inaccuracies in estimating fraction absorbed based on small molecular weight transport studies as such processes can be saturated and contain a nonlinear dose dependent permeability values. Additional studies into the apical-basolateral (AB) versus basolateral-apical (BA) $P_{app}$ need to be performed in order to evaluate the apparent *intrinsic* permeability of the dendrimers, especially since differential AB and BA $P_{app}$ has already been observed in Caco-2 monolayers.[10] Therefore the above predicted values for oral absorption can potentially be highly biased and realistically can only be ascertained when administered to human subjects. Overall a predicted fraction absorbed for 13-14 kDa dendrimers of 30% in humans is relatively large when compared to the fraction absorbed of 4 kDa PEG of 0.4%, and may be an overestimation[29,51] (Table 5.2).

Yet even a low fraction absorbed of <10% can be effective for polymeric

**Table 5.2.** Comparison of $P_{app} \times 10^{-6}$ and % absorbed of various macromolecules in literature and this study ([a]estimated values).[24,29,60–62]

| | | Jejunum | Colon | % Absorbed |
|---|---|---|---|---|
| This Study | Mannitol | 3.07 | 5.90 | |
| | G3.5-FITC | 2.10 | 3.65 | 35[a] |
| | G4-$NH_2$-FITC | 0.96 | 2.08 | 11[a] |
| | 4kDa Dextrans | 0.51 | 1.27 | |
| Literature data | Mannitol[60,62] | 5.56 | 4.95 | 38 |
| | Ovalbumin[60] | 0.02 | 0.02 | 0.1[a] |
| | α-lactalbumin[60] | 0.11 | 0.01 | 0.1[a] |
| | 4 kDa Dextrans[60] | 0.15 | 0.10 | 0.1[a] |
| | 70 kDa Dextran[60] | 0.01 | 0.01 | 0.1[a] |
| | 4 kDa Poly(ethylene glycol)[29] | 0.88 | | 0.4 |

drug delivery, especially if the attached drug is highly potent and the biodistribution can be modified to passively or actively target the site of action.[54,55] This research focus has been explored by our lab and others.[56–58] For example, G3.5-SN38 compounds have been previously synthesized.[56] If one were to estimate the blood concentration of dendrimer-drug conjugates, based on a modest oral dose of 2 mg/kg in a 70 kg human with a fraction absorbed of 30% this would give an estimated serum concentration of 0.6 µM (42 mg of G3.5-SN38, in 5L plasma volume). This matches the previously reported $IC_{50}$ value of G3.5-SN38 in HT-29 cell cultures.[7] These results show that dendrimer drug conjugates could potentially be therapeutically effective as an oral drug delivery system for highly potent drugs with $IC_{50}$ values less than 1 µM.

## 5.6 Conclusion

The permeability of PAMAM dendrimers across intestinal epithelial barrier models has been evaluated in Caco-2 monolayers, [8,10,13–15] isolated rat intestinal tissue,[3,38] in vivo to rats[42] and mice[1,6] and in isolated human tissues as reported here. Of note was the permeability of PAMAM dendrimers ranging from $2.1\times10^{-6}$ for G3.5 probes in isolated human jejunum. The permeability of PAMAM dendrimers is 4 fold higher than other 4 kDa dextran tested in human isolated jejunum, and has an estimated fraction absorbed 100 fold higher than 4 kDa PEG polymers.[29,51] The regional dependence of G3.5 and G4 PAMAM dendrimer transport has been established, with demonstrated highest rate of transport in jejunal segment. Comparison of human jejunal transepithelial transport with rat

and Caco-2 monolayers has specifically demonstrated an overestimation of dendrimer $P_{app}$ by isolated rat and Caco-2 models.

The change in intestinal morphology caused by PAMAM dendrimers appears to correlate with previous in vivo toxicity studies, but not with previous Caco-2 models. This potentially provides a dosing window of opportunity where dendrimer drug delivery can be developed.[6,42] Specifically an estimated MTD of 1000-3000 mg/kg is implied by the changes in epithelial morphology noted at 10mM concentrations in human isolated tissue.

Despite residing in the BCS class for low permeability, the attachment of highly potent drugs may still benefit from the enhanced permeability that PAMAM dendrimers can offer. The potential localized release of PAMAM dendrimer-drug conjugates could enhance efficacy and safety of the drug, while simultaneously providing an oral formulation. Future developments utilizing PAMAM dendrimers for oral drug delivery may focus on attaching highly potent drugs that otherwise have dose limiting toxicity or solubility.

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# CHAPTER 6

# CONCLUSIONS

## 6.1 Oral drug delivery

In this dissertation, PAMAM dendrimers were evaluated as nanocarriers for delivery via the oral route. The hypothesis in the first part of this dissertation was that PAMAM dendrimer permeability through isolated rat intestinal tissue was significantly higher than similar sized dextran molecules and free fluorescein isothiocyanate (FITC). This result was confirmed in the study with generation 3.5 (G3.5) PAMAM dendrimer transport being significantly greater than free FITC.[1] G3.5 and G4 PAMAM dendrimer transport trended higher than 4 kDa FITC-dextran as well confirming their appreciable permeability in isolated rat intestinal tissue.

Corollary to the first hypothesis was that 1 mM concentrations of PAMAM dendrimer would not cause significant epithelial damage to isolated rat intestinal tissue. Previous work in Caco-2 models showed significant difference in concentration and incubation time dependent effects on epithelial morphology between Caco-2 and in vivo models.[2–5] This result was confirmed by two separate studies utilizing supratoxic Caco-2 concentration of PAMAM dendrimers and observing their relative lack of intestinal damage in isolated tissue. This

result confirms that Caco-2 cells are more sensitive to PAMAM dendrimer induced changes in intestinal morphology than Caco-2 or in vivo models.[1,6]

This result led to the evaluation of PAMAM dendrimer concentration at which morphological changes occur in isolated intestinal tissue. Previously the maximum concentrations observed at which morphological changes and lactate dehydrogenase release did not occur were less than 0.1 mM for G4 PAMAM dendrimers and less than 1mM for G3.5 dendrimers (120 min incubation times).[5,7] This result was found to be significantly higher in isolated rat intestinal tissue, where neither G3.5 nor G4 dendrimers caused morphological changes until 10 mM concentrations were reached.[6] This difference was assumed to be a function of the difference between Caco-2 and isolated tissue models including mucous layers, enzyme expression and morphological properties.

Aim 2 of this dissertation focused on the regional difference between PAMAM dendrimer transport in isolated rat intestinal tissue. Colon and jejunum samples were observed to have differential rates of transport for PAMAM dendrimers. G3.5 and G4 PAMAM dendrimer transport was significantly higher in isolated jejunal mucosae than in colonic mucosae.[6] This is not surprising considering the thicker mucous and reduced surface area of the rat colonic epithelium.[5,6] Interestingly, the permeability in rat intestinal jejunum was found to have comparable results with Caco-2 studies, but not with rat colonic permeability. Caco-2 cells are generally considered to have characteristics of small bowel similar to the large intestine.[8]

The difference between Caco-2 barriers and isolated tissue may be due to

the physiological factors mentioned previously and also the reduced thickness of single monolayers compared to isolated intestinal tissue. Further analysis of the comparison between Caco-2 and rat isolated intestinal epithelium noted key differences in TEER and mannitol permeability in the presence of PAMAM dendrimers. TEER was significantly reduced in Caco-2 models at 0.1 mM but no effect was observed at 1mM concentrations for G4 dendrimers.[5,6] The influence of dendrimers on mannitol permeability was found to be significant at 0.01 mM for G4 dendrimers in Caco-2 cells, whereas concentrations as high as 1 mM caused no change in isolated rat intestinal tissue. G3.5 dendrimers caused an increase in mannitol permeability at 0.1 mM in Caco-2 cells, but were not found to cause a statistically significant increase in permeably at 10 mM in isolated intestinal tissue. This confirms the conclusion that PAMAM dendrimers are more sensitive to penetration enhancement than isolated or in vivo gastrointestinal physiology and adds evidence that penetration enhancing effects observed in Caco-2 monolayers should be treated with caution.

The third aim of this dissertation was to address the permeability of PAMAM dendrimers in isolated human tissue and to predict a human fraction absorbed based on previously published correaltins.[9] This resulted in the observation that G3.5 PAMAM dendrimer permeability through isolated jejunal tissue was $2.1\times 10^{-6}$ cm/s. The estimated fraction absorbed for such a rate is ~30%. This result is very high for a macromolecule of 12.9 kDa molecule and may be an overestimation since previous correlations were established for small molecular weight drugs and have not been developed for macromolecules which

are transported via endocytic routes and interact with the barrier to open tight junctions. Nevertheless this rate is significant especially when comparable size PEG molecules have a human fraction absorbed of 0.4%.[10,11] While this does not allow PAMAM dendrimers to function as an oral drug delivery agent for all types of pharmaceuticals, it does allow the oral delivery of highly potent compounds. Highly potent cancer drugs may benefit from PAMAM dendrimer drug delivery, drugs that with an $IC_{50}$ greater than 1μM are predicted to not reach a viable blood concentration for therapeutic delivery

The comparison of PAMAM dendrimer permeability between Caco-2 rat and isolated tissue models led to the conclusion that Caco-2 and rat models generally overestimate human absorption in the jejunum epithelium for PAMAM dendrimers. Dextran transport was noted to be similar between human, rat and Caco-2 models, showing that the compact molecular architecture of PAMAM dendrimers may have significant impact on biodistribution and oral absorption. This result has been confirmed previously by head to head studies with *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymers and PAMAM dendrimers.[12] PAMAM dendrimer permeability and oral absorption may be facilitated by their compact structure and smaller hydrodynamic radius than other linear polymers, in addition to opening tight junctions and cellular uptake by endocytosis.

This research did not include any drug molecules in the studies of transepithelial transport. This impacts this research in two ways. First, since attachment of drug molecules could potentially alter transport, avoiding them aided in understanding the innate ability of PAMAM dendrimers to traverse the

intestinal barrier. Understanding the transepithelial transport of PAMAM dendrimers was a necessary precursor to understanding the positive (or negative) effects of drug attachment on permeability. Second, avoiding the use of a drug in these studies decreased the impact on clinical drug delivery. Previous research has observed the ability of PAMAM dendrimers to increase drug transport through the intestinal epithelium as mentioned in Chapter 2, so this was not the primary focus of this dissertation.[2,13]

Based on this research in isolated human intestinal tissue it is estimated that drugs that have an $IC_{50}$ <1µM should be investigated for oral drug delivery. Estimations from this study show that PAMAM dendrimer transport may be capable of delivering a drugs at an appreciable rate, but probably not sufficient for low potency compounds with an $IC_{50}$ >1µM.

## 6.2 Future directions

Future directions of this research include studying intestinal transport of PAMAM dendrimers through isolated tissue in a dose depended manner and verifying their transport in the apical to basolateral and basolateral to apical direction. This would allow a greater understanding of the maximum permeability of PAMAM dendrimers and key insight into the serosal transport properties of dendrimers (whether active or passive).

Attempts should be made to attach highly potent ($IC_{50}$ <1µM) small molecular weight drugs for oral delivery. This will significantly enhance the likelihood of clinical success since blood concentrations of dendrimers are not

likely to exceed 1μM. Efficacy and safety of these systems will have to be evaluated on a case by case basis to assure reduced toxicity of polymeric delivery does not also reduce the efficacy of the attached therapeutic agent and diminish the effect of the treatment.[14] Therapies that are high on the list of potential candidates for drug delivery include highly potent anticancer drugs such as SN38, docetaxel and gemcitabine. Some of these drugs are administered via 10 h or more infusions for patients. Thus an oral formulation of these drugs would strongly benefit patient quality of life.

Future studies may evaluate a range of penetration enhancers to find the best match for PAMAM dendrimer oral drug delivery.[15] These studies should be carried out in isolated tissue models to assure realistic values of $P_{app}$ are obtained. Combinatorial studies in isolated tissue may be necessary to evaluate the wide spectrum of penetration enhancers available. Enhancers that do not damage the intestinal morphology, do not sequester dendrimers, and aid in enhancement will be ideal in order to find a safe and effective delivery strategy.

In order to carry out studies in isolated tissue at a higher rate, high throughput Ussing chambers may be of use. Both the sheer volume required for standard Ussing chambers and format of the tissue chambers for mounting the tissue was found to be limiting. A potential high throughput, low volume Ussing chamber would greatly enhance this study and allow for much more rapid analysis of the hypothesis posed. Comparative studies would be required between any new models and current practice to assure that proper correlations were observed.

Drugs that have an $IC_{50}$ <1μM should be investigated for oral drug delivery. Estimations from this study show that PAMAM dendrimer transport may be capable of delivering drugs at an appreciable rate, but probably not sufficient for low potency compounds with an $IC_{50}$ above 1μM.

Future studies should specifically focus on the attachment of SN38, gemcitabine or docetaxel to PAMAM dendrimers for the development of oral drug delivery. Docetaxel is already used to treat many patients, so adding a novel oral formulation to the possible routes of administration could be potentially benefitial. Additionally there already exist industry partners developing Priostar™ dendrimers for intravenous therapy of docetaxel. Their promising results from phase I clinical trials may be helpful for establishing dendrimer oral drug delivery although the chemical composition does not exactly match.[16–18]

Bioconjugation strategies for drugs with PAMAM dendrimers may focus on the critical step where drugs are attached to dendrimers. Attaching various linker molecules has not been a major issue due to the similarity in polarity of the linkers and dendrimers. Generally the drugs used do not have the same polarity as dendrimers and thus have made finding an appropriate solvent system difficult. Future research may focus on DMF soluble drugs. Additionally HBTU activation may be better able to activate –COOH than carbodiimide activation agents for bioconjugation. Achieving the right drug-dendrimer molar ratio will be important so as to still remain soluble in aqueous systems (too much drug attachment may cause the dendrimer to precipitate in water).

Following drug attachment in vitro cytotoxicity should be tested. Target

cancer cell lines and intestinal epithelial cell lines would be primary targets for cytotoxicity assays. The cytotoxicity studies should focus on testing $IC_{50}$ values in these models.

Following cytotoxicity evaluation the transepithelial transport in Caco-2 cells, isolated rat and isolated human tissue should be observed to assure the permeability of dendrimer drug conjugates is still sufficient for oral drug delivery. Attachment of drugs could change the rate of absorption.

These studies should be followed by in vivo toxicity and efficacy studies in mice or rats. Penetration enhancers may be required to achieve efficacy, and potentially sodium caprate or other commonly used enhancers could be used to achieve efficacy in animals. These combinations would also need to be checked for toxicity in the same model systems at the same dosages as used in efficacy. Potentially the dendrimer drug system will not require a penetration enhancer and efficacy would be sufficient without it.

The critical problems that need to be resolved for clinical development of PAMAM dendrimer drug conjugates are:

1) PAMAM dendrimers have a wide batch to batch variability requiring characterization and purification of every lot. This inconsistency would be unacceptable for a clinically approved candidate in terms of reproducibility and good manufacturing practice.[19] While much has been done to improve dendrimer synthesis, novel strategies using microwave assisted synthesis may improve the kinetics of dendrimer reactions (that normally take days to weeks to complete) and may be able to improve lot to lot variability. Alternatively a real approach

would be to purify in bulk all compounds received on an industrial scale to remove all smaller generations and other impurities.

2) Dendrimer conjugation with drug molecules has had inconsistencies that have made attachment of drugs difficult. Low drug loading and a lack of appropriate solvents for solubilization of dendrimer-drug reactions have made the synthesis exceedingly difficult. This problem may be offspring of the previously mentioned dendrimer batch to batch variability, but has not been resolved fully, and may be a function of the altered pKa of surface amine or carboxylic acid groups as mentioned in Chapter 2. The attachment of hydrophobic molecules to PAMAM dendrimers has been difficult to reproduce consistently and may need to be optimized in order to create a clinically viable macromolecule. This problem has been noted by other groups as well as our own.[20] Reproducible synthetic methods for drug attachment is critical for consistent patient care and thus is required by the Food and Drug Administration for entrance into clinical trials. Other moieties such as imaging agents and targeting ligands will require the same characterization and reproducibility in order to facilitate clinical translation.

3) Finally for a full scale clinical study to commence, good laboratory practice and good manufacturing practice must be implemented in a preclinical pharmacokinetic, safety and toxicity study. These studies must include absorption, distribution, metabolism, teratogenicity, efficacy, clearance and long term carcinogenic effects. Rats, mice and nonhuman primate models may be suitable for preclinical testing of these parameters to develop a safety and efficacy profile. These studies are necessary to advance dendrimer drug delivery

to a clinical stage where human efficacy can be tested.

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# APPENDIX

# EFFECT OF PEPTIDE PENETRATION ENHANCERS ON INTESTINAL PERMEABILITY OF PAMAM DENDRIMERS

## A.1 Introduction

In spite of initial results showing appreciable permeability of PAMAM dendrimers across rat epithelium, the human intestinal permeability was found to be significantly lower,[1] limiting dendrimer drug delivery to highly potent drugs.[1] While this level of delivery is significant, it is not sufficient for delivery of less potent drugs which require a larger fraction absorbed.[2] In order to increase the therapeutic impact of PAMAM dendrimer oral drug delivery we explored the use of penetration enhancers for improving dendrimer permeation across the intestinal epithelium.

Specifically we have looked into the physical mixtures of the P640 peptide penetration enhancer with the structure RRVEVKYDRRKKR (one letter amino acid sequences used) with dendrimers. This sequence investigated by Dr. Randall Mrsny of University of Bath is a myosin light chain phosphatase inhibitor.[3–7] Its mode of action is through inhibiting the dephosphorylation of myosin light chain (MLC) which proceeds to cause contraction of the actin

myosin cytoskeleton and opening of the tight junction pores.[7,8]

## A.2 Experimental methods

### A.2.1 Materials

Reagents for solid phase amino acid synthesis were obtained from AAPTEC (KY, USA). Fluorescein isothiocyanate (FITC) (cat. no. F7250), fluorescein isothiocyanate dextrans (cat. no. FD10S), and Canadian origin fetal bovine serum (FBS) were obtained from Sigma-Aldrich (MO, USA). PAMAM dendrimers (G3.5-COOH, and G4.0-NH2) were obtained from Dendritech, Inc. (Michigan, USA). $^{14}$C mannitol (0.1mCi/mL in sterile water) was obtained from American Radiolabeled Chemicals (MO, USA). Cells were obtained from American Type Culture Collection (MD, USA). All other chemicals were obtained from VWR.

### A.2.2 Methods

6.2.2. Synthesis and characterizations

RRVEVKYDRRKKR sequence was synthesized using solid phase peptide synthesis. Briefly to a preswelled Arginine-loaded Wang resin (0.3mmol/gram loading) was added a solution of 20% v/v piperidine in anhydrous amine-free dimethyl formamide (DMF). Deprotection was carried out for 30 min, followed by 3 washes with DMF. Subsequently to a Fluorenylmethyloxycarbonyl (FMOC) protected L-lysine was added 2.5 equivalents of O-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) in a solution of 0.4 M N-

methylmorpholine (NMM) in DMF. The FMOC-lysine was fully dissolved and then added to the arginine loaded Wang resin. The resin and the FMOC-lysine were stirred on a rotary shaker for >4 h followed by 3 washes with DMF and then testing via the Kaiser test for the presence of free amines. An absence of purple color was indicative of the completion of the coupling of the reaction. Subsequent deprotection and coupling were performed in a repetitive fashion as previously outlined to obtain the oligopeptide. Final deprotection was carried out for 16 h using a cleavage cocktail of 95% TFA: 2.5% H20:2.5% diisopropylsilane (v/v). The crude peptide was precipitated in cold diethylether and washed several times with diethylether. The final product was purified via preparative high pressure liquid chromatography (HPLC) at a flow rate of 15mL/min on a Zorbax C18 column using an isocratic flow of acetonitrile: water (20:80) with 0.1% TFA. The final product was tested for purity using analytical HPLC (5µm C18 at 1mL/min flow rate) using an isocratic acetonitrile: water (20:80) with 0.1% TFA.

The purity of the peptide was monitored by matrix absorption laser desorption ionization time of flight (MALDI-TOF) mass spectrometry using a Bruker's ultrafleXtreme™ MALDI-TOF/TOF (AZ, USA). Samples were spotted using dried-droplet method. A solution of saturated 2′,4′,6′-trihydroxyacetophenone monohydrate (THAP) in a solvent of 50:50 water: acetonitrile 0.1% TFA was prepared by mixing the matrix powder with 0.5 mL of solvent, and then centrifuged to pellet of undissolved THAP. The supernatant was used for sample preparation for MALDI analysis. Samples (0.5 µL of 1 pmol/µL) were loaded onto a stainless steel target plate and mixed on the target

with 0.5 µL of supernatant of saturated matrix solution. Then the sample spot was dried, the spot was ablated with a 1 kHz smartbeam-II™ laser technology while the sample was simultaneously desorbed, and then accelerated into a flight tube. The MALDI spectrum was acquired in linear mode, at a mass range from 1000 to 120,000 Da.

### A.2.2.1 FITC labeled dendrimer synthesis

FITC labeled conjugates were prepared as reported previously.[9] The conjugates were characterized for absence of free FITC using Superose 6 column at a flow rate of 1 mL/min on a fast protein liquid chromatography system (FPLC). FITC loading was assessed by spectrophotometry as described previously.[10]

### A.2.2.3 Caco-2 cell culture and permeability assays

Caco-2 cell cultures were prepared according to previously published methods.[9,11] Cells of passage 6-16 were grown at 37°C in a tissue culture incubator at 5% $CO_2$ atmosphere in air with 95% relative humidity. Dulbecco's Modified Eagle's Medium (DMEM) was used with 10% fetal bovine serum (FBS). The media was changed every two days until 90% confluence was attained and then passaged using trypsin-like enzyme (Gibcolife, NY, USA). Cells were passaged at least twice using trypsin-like enzyme after removal from storage in liquid nitrogen before being seeded onto Transwells® plates. Seeding was done on 24-well polyester Transwells® (Sigma Aldrich, cat. no. CLfigure470-48EA) at

a density of $2.6\times10^6$ cells/$cm^2$. Cells were allowed to grow for 21-29 days before being used for transport experiments. Prior to permeability assays DMEM media was removed and transport media was applied to the apical and basolateral surface. Transport medium consisted of Hank's balanced salt solution (HBSS) supplemented with 10 mM *N*-(2-hydroxyethyl)piperazine-*N´*-Poly(amido amine) (2-ethanesulfonic acid) hemisodium salt (HEPES) buffer at pH 7.4.

Transport experiments were carried out after cells were equilibrated with media for 60 min. At t=0 min, the transport buffer was decanted and treatment was added to the apical side. FITC dextrans (5mg/mL) and FITC-G3.5 and G4 dendrimers (0.1 mM) and various concentrations of P640 peptide were added to the apical side of the cells. Monolayers were then incubated at 37°C on an orbital shaker at 350 RPM (G76, New Brunswick, NJ, USA). 200 µL aliquots were sampled every 30 min from the basolateral side starting at t=0 for 120 min and the volume was replaced by warmed transport buffer. Temperature was maintained at 37°C while added media, measuring transepithelial electrical resistance (TEER) or sampling the solution by using a warm plate set at 37°C. This was done in order to obtain accurate TEER measurements due the temperature dependence of the measuremnt.[12] TEER was measured using Chopstick electrode set (World Precision Instruments, FL, USA).

Permeability of FITC-dextrans and FITC labeled dendrimers was quantified via fluorescence intensity appearing on the basolateral side of the cell monolayer. Fluorescent samples were quantified using an excitation wavelength of 495 and emission wavelength of 525 nm on spectrophotometer (Spectramax

M2, Molecular devices, CA, USA). All experiments included 5µL of $^{14}$C-mannitol as an internal control, which was quantified using liquid scintillation (LS-6000IC, Beckman, CA, USA).

A.2.2.5 Human isolated intestinal tissue transport

Healthy intestinal tissue was obtained from surgical patients immediately after removal as reported preiously.[1] The tissue was macroscopically analyzed by a pathologist for necrotic or cancerous lesions, cut and immersed in ice cold oxygenated Krebs-Hensleit (KH) buffer at pH 7.4. The tissue was then immediately transported to the lab for mounting in Ussing chambers. Removal of the serosal muscle layer was performed in an oxygenated tissue dissection basin. After the external muscle layer was removed the epithelium was mounted in Ussing chambers with an exposed surface area of 0.3 cm$^2$ (Physiolgic Instruments, CA, USA). Chambers were filled with 5mL of 37°C KH buffer gassed with 95% $O_2$/5% $CO_2$. Tissue was allowed to equilibrate in Ussing chambers for 45 min to allow for achievement of maximum initial TEER. At time t=0 min solutions containing test probes were added to the apical side of the chambers.

Treatments included 4 kDa FITC-labeled dextrans (FD4) at 5mg/mL and p640 peptide at concentration noted. All experiments included 5µL of $^{14}$C-mannitol as a marker of paracellular permeability added to the apical side of the tissue. The apical and basolateral side of the chambers were sampled as mentioned previously.[1] Fluorescence and radioactivity were quantified as above.

TEER was taken using Evom-2 TEER measurement device (Warner

Instruments, Sarasota, FL, USA) attached to AgCl electrodes (Physiologic Instruments, CA, USA) that were previously filled with a 3% agar solution made in 3M KCl. TEER measurements were taken 15, 10 and 0 min before treatment and every 5 min after treatment began for the initial 20 min. Following the initial 20 min, TEER was measured every 20 min for 2 h. TEER results were analyzed using a student's t-test to compare treatment values to the control.

#### A.2.2.6 Statistical analysis

Statistical analysis of the $P_{app}$ data was compared using ANOVA, with Tukey's post analysis in GraphPad® Prism (version 6.0c, CA, USA).

## A.3 Results and discussion

The goal of this study was to enhance the permeability of PAMAM dendrimers across the intestinal mucosa. For this reason we explored the use of the P640 peptide. The synthesis of the P640 peptide resulted in a product with 99% purity by HPLC analysis and a single major peak via MALDI-TOF analysis (Figure A.1). The product was soluble in aqueous buffers.

TEER results from Caco-2 studies were on average 127 ± 16 Ω-cm$^2$ at the beginning of the experimentation. P640 peptides caused a trending increase in TEER at 60 min time points. This trend later was reversed for 40mM P640 groups where a significant decrease in TEER was noted, but not for 20 mM and 5 mM P640 groups which still remained above control values (Figure A.2). This result was unexpected since penetration enhancers generally cause a transient

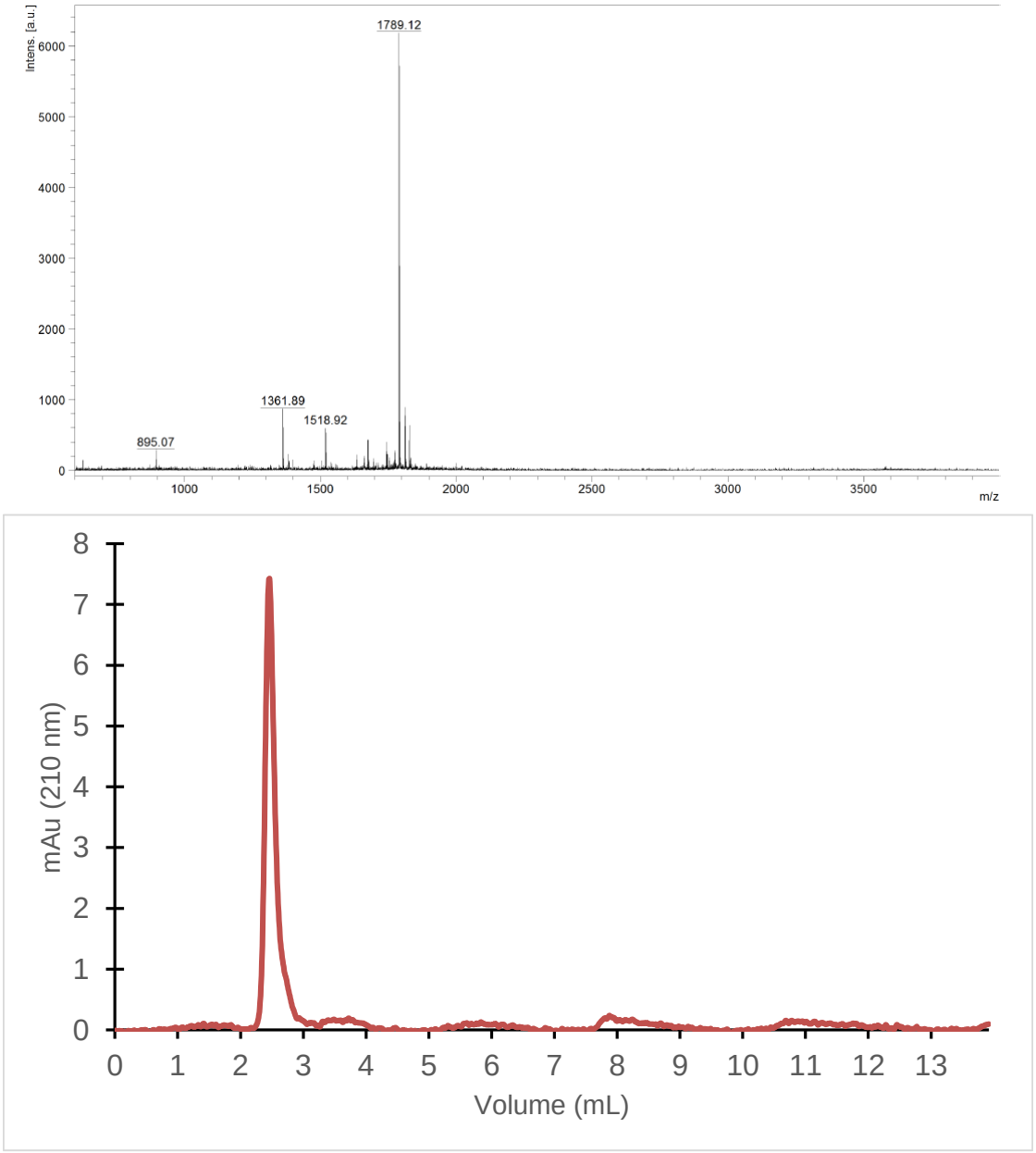


**Figure A.1** MALDI-TOF spectra and HPLC analysis of P640 peptide. MALDI-TOF analysis showed a single major peak at M/Z 1789 (A). Predicted molecular weight was MW 1789. HPLC chromatogram of the purified peptide product had a purity of 99% (B).

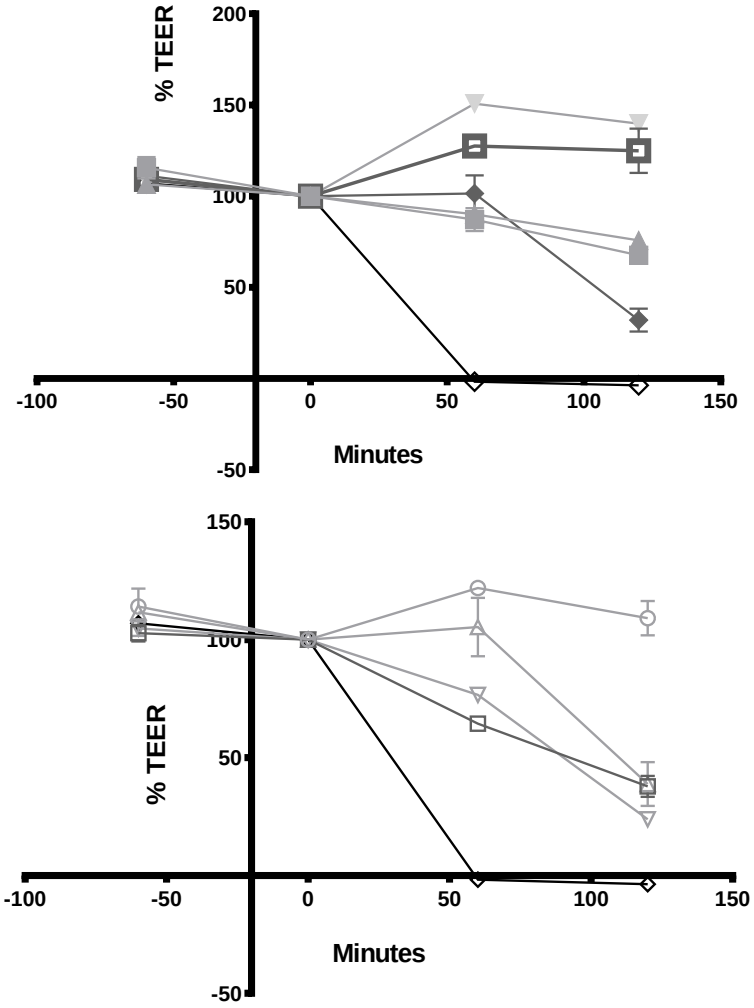


**Figure A.2.** Caco-2 TEER results with 4 kDa FITC dextran (FD4) and G3.5 and G4 dendrimers in the presence of P640. Upper panel: control, FD4 + p640 5 mM, FD4 + p640 20 mM, FD4 + p640 40 mM, Triton X100. Caco-2 TEER results. P640 peptides mixed with 4 kDa FITC dextran (FD4) caused a trending increase in TEER at 60 min time points. This trend was reversed at later time points for 40mM P640 groups, which were observed to drop in TEER ($p<0.05$). Lower panel: G3.5 0.1mM. G3.5 0.1 mM + P640 40 mM, G4 0.1 mM, G4 0.1 mM + P640, Triton X100. 40 mM. 40mM P640 peptides combined with 0.1mM PAMAM dendrimers caused a reduction in TEER compared to control , G4 and G3.5 treatments ($p<0.05$).

(or permanent) reduction in TEER.[13] Forty mM P640 caused a reduction in TEER at 120 min time points ($p<0.05$), but the increase in TEER for lower concentrations (i.e., 5 mM, 20 mM) did not match the expected results.

Dendrimers mixed with 40mM P640 peptides caused a decrease in TEER ($p<0.05$). G3.5 dendrimer with P640 was statistically reduced compared to 0.1 mM G3.5 dendrimers at 120 min. This reduction was also significant for P640 with 0.1 mM G4 dendrimers, but the difference was much less. The cause of this variation between dendrimer G3.5 and G4 may be due to the charge induced TEER reduction native to G4 dendrimers. G4 dendrimers are known to have significant TEER reducing effects in Caco-2 cells even at 0.1 mM concentrations.[13,14] Anionic G3.5 dendrimers at 0.1 mM concentrations have been observed to have lesser effect on TEER than their cationic counterparts. This is probably due to enhanced tissue association of cationic dendrimers with anionic cell surfaces.[14]

Surprisingly G3.5 and G4 PAMAM dendrimer transport was reduced in the presence of 40 mM P640 peptides (Figure A.3). This result was true for both G3.5 and G4 PAMAM dendrimers, although the reduction was not statistically significant. This result was counterintuitive since previously it was reported that arginine rich peptide sequences have penetration enhancing properties.[15] In mechanistic studies oligo arginine has been found to interact with associated macromolecules and this association was critical for their penetration enhancement.[16] Surprisingly both cationic G4 PAMAM dendrimers and anionic G3.5 dendrimer $P_{app}$ were reduced in the presence of the P640 peptide indicating

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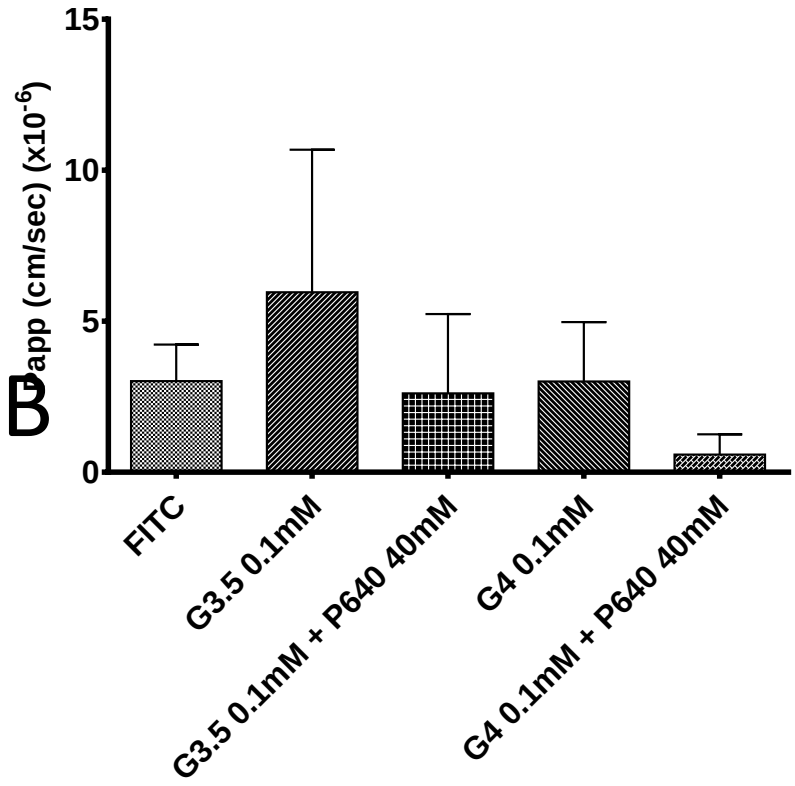


**Figure A.3.** Caco-2 dendrimer permeability in presence of P640. G3.5 and G4 PAMAM dendrimer transport trended toward a decreased permeability in the presence of P640 peptides.

a lack of association for positively and negatively charged dendrimers.

This phenomenon does not explain the reduction in transport observed. The reduction may be due to an unknown physiological mechanism induced by the P640 peptide. Potentially P640 peptides may saturate an endocytic pathway which PAMAM dendrimers themselves are transported by.[17] It has been observed that endocytosis inhibitors in Caco-2 transepithelial transport studies inhibited dendrimer permeability across the membrane. P640 peptides may have similar effect by outcompeting dendrimers for endocytic vesicles. Nonetheless this requires further evidence since the mechanism of internalization of arginine rich sequences is not entirely understood and continues to be a topic of discussion in the scientific community.[18]

$P_{app}$ of 4 kDa FITC-dextrans in the presence of P640 peptides in Caco-2 monolayers was similarly not significantly increased (Figure A.4). In spite of 40 mM concentrations the permeability only tended to increase. This led to the conclusion that, at least in Caco-2 models, P640 peptides are not effective as a penetration enhancer. This result was confirmed by mannitol permeability results which showed no statistically significant increase in permeability at 40mM concentrations of P640 peptide with or without PAMAM dendrimers. (Figure A.5, A.6)

Caco-2 models have significant differences from animal or human tissues.[1,9,19,20] Indeed mucous layers, tissue morphology, heterogeneous cell types and other factors impact the permeability of PAMAM dendrimers and other probes such as mannitol. For this reason further investigation of P640 peptides

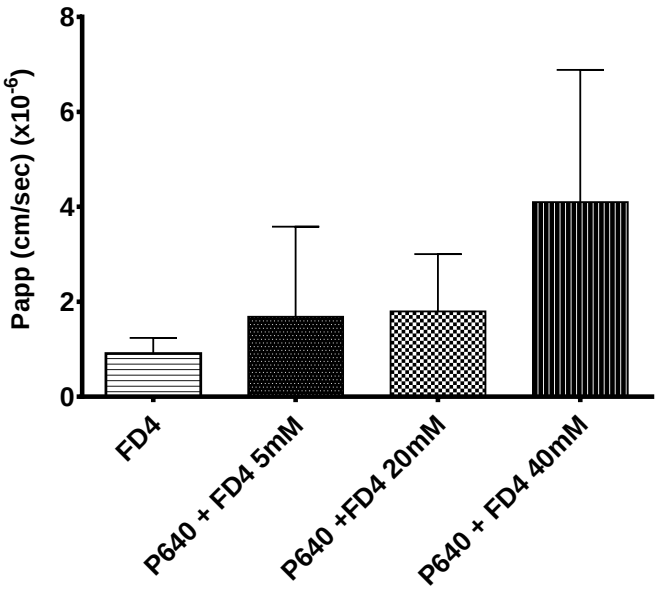


**Figure A.4.** Caco-2 permeability of 4 kDa FITC dextran (FD4) in presence of P640. FITC dextran permeability was not statistically increased at 40mM concentrations of P640 peptide.

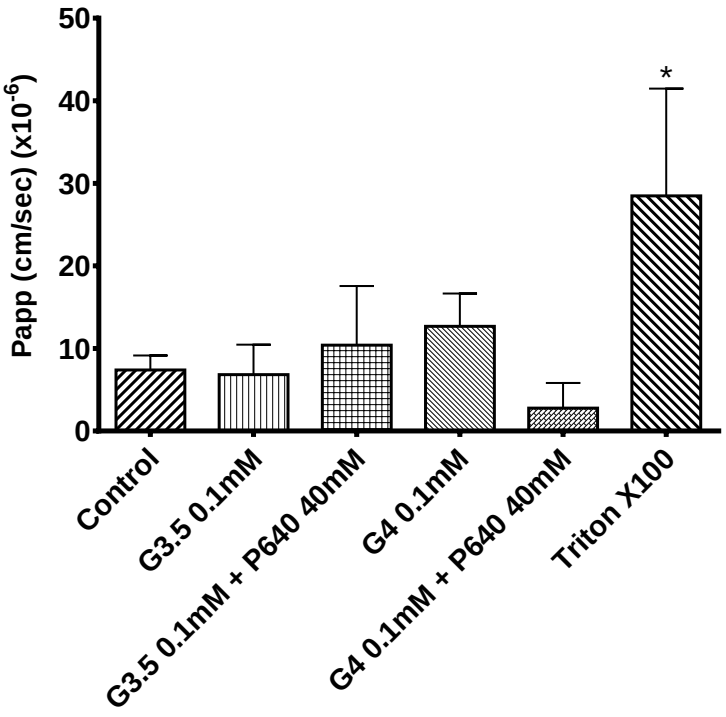


**Figure A.5.** Caco-2 mannitol permeability with dendrimers and P640. Mannitol permeability was enhanced with the positive control Triton X100, but not in the presence of 0.1 mM G3.5 and G4 dendrimers or the combination of dendrimers with 40mM P640. (* signifies $p<0.05$ from control)

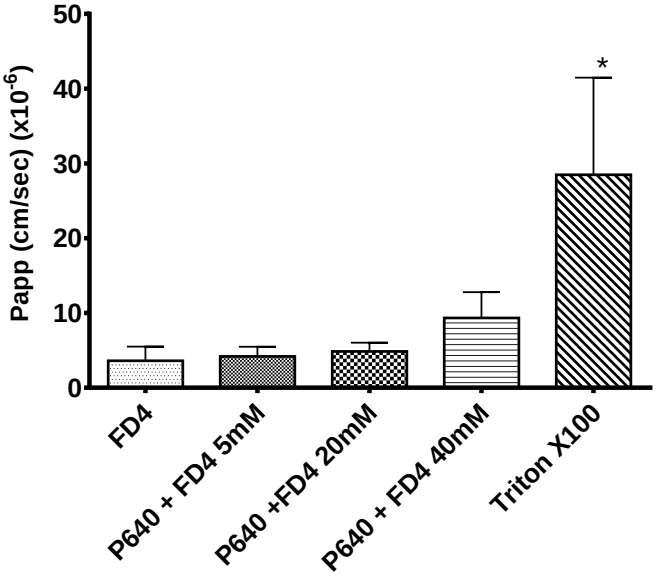


**Figure A.6.** Caco-2 mannitol permeability in presence of 4 kDa FITC dextrans (FD4) and P640. P640 did not cause a statistically significant increase in mannitol permeability compared to control. (* signifies $p<0.05$ from control)

was carried out in human isolated tissues obtained from patients.

TEER results from these studies showed no significant reduction with treatments up to 20 mM of P640 peptides (Figure A.7). These results correlated well with FITC dextran transport (Figure A.8) and mannitol transport (Figure A.9) which also showed no significant increase in permeability. Indeed the permeability of FITC dextran appeared to drop at higher concentrations of P640 peptide indicating a lack of response to P640 peptide penetration enhancer. This result further confirmed the inability of P640 to mediate increased intestinal permeability at the dose and incubation period observe in this study.

## A.4 Conclusion

The results of this study indicate that P640 peptides do not mediate enhanced permeability of PAMAM dendrimers in Caco-2 cells at 120 min incubation times and 40 mM concentrations. Further work at longer timeframes may show a different response depending on the stability and uptake of these sequences. A greater understanding of PAMAM-peptide interactions and the internalization mechanisms and response time of these sequences may be necessary to confirm their status as a peptide penetration enhancer in Caco-2 cells for PAMAM dendrimers.

Furthermore the addition of P640 peptides to human isolated colonic epithelium did not cause a significant enhancement in 4 kDa FITC-dextran and mannitol permeability indicating that future work may be needed to optimize the sequence and improve the binding affinity to MLCK phosphatases. Most

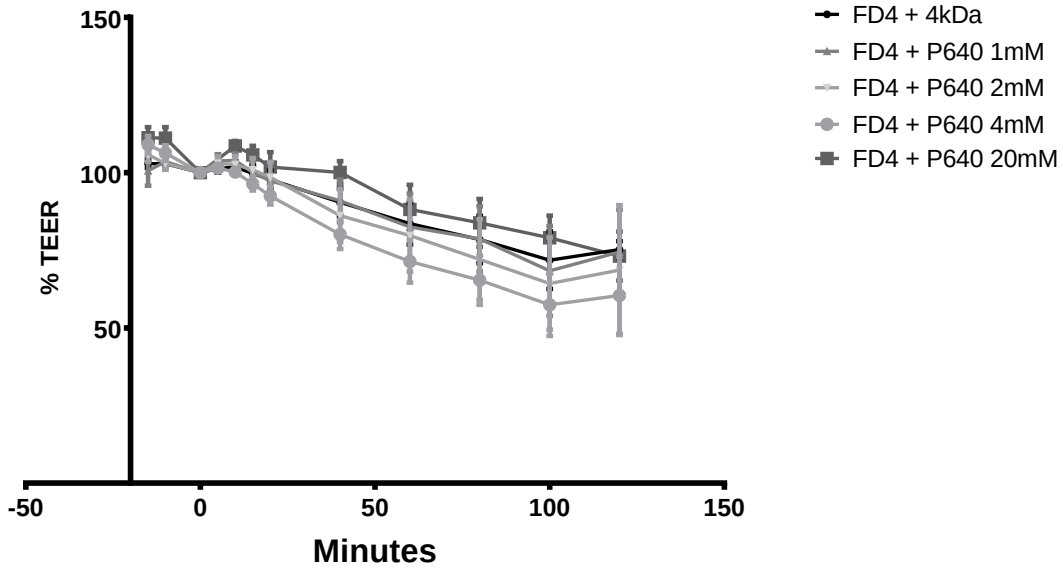


**Figure A.7.** Isolated human colon percent TEER in presence of P640 and 4 kDa FITC dextrans (FD4). P640 did not cause a decrease in TEER at 20 mM concentrations compared to control.

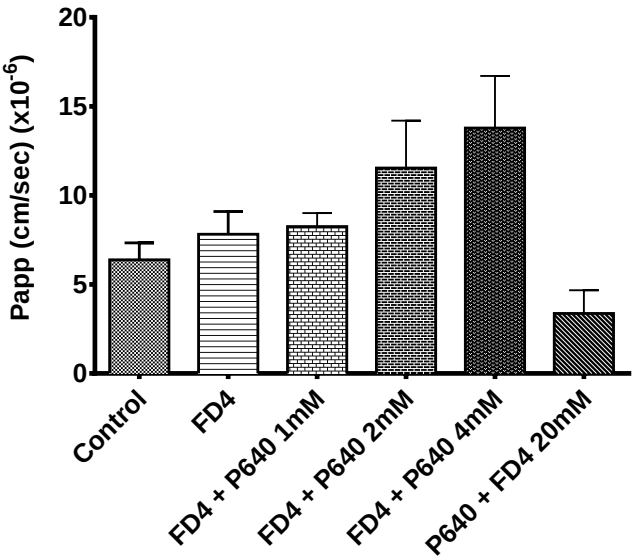


**Figure A.8** Isolated human colon permeability of control (FITC) and 4 kDa FITC dextran (FD4) in the presence of P640 peptides. P640 showed a trending decrease in FD4 permeability at 20 mM concentrations.

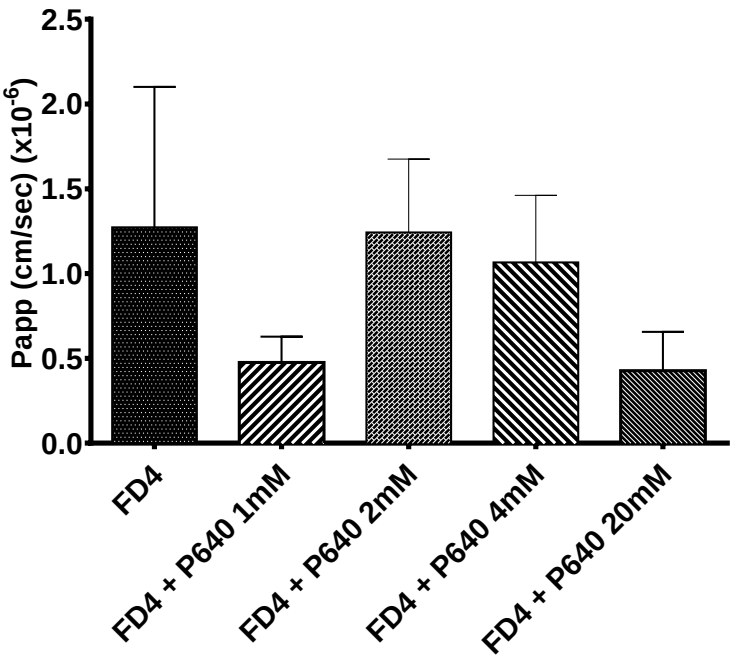


**Figure A.9.** Isolated human colon mannitol permeability in the presence of 4 kDa FITC dextrans (FD4) and P640. The permeability of mannitol in the presence of FD4 and P640 peptides trended to decrease.

importantly the potential interaction of the dendrimers with the peptide penetration enhancers and the subsequent effect they may have on the permeability values observed need to be investigated.

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