EVALUATION OF DENDRIMERS AS A POTENTIAL ORAL DRUG DELIVERY SYSTEM

by

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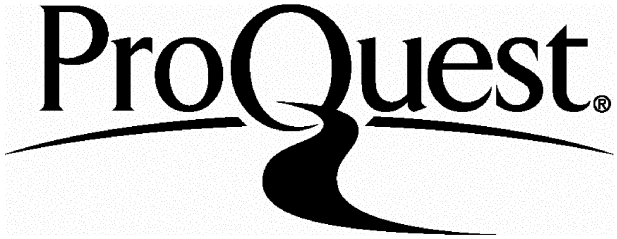

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Abstract

Dendrimers are novel nanoscale sized macromolecules that have a well-defined highly branched structure and a large number of reactive chain-ends. Here the potential use of dendrimers as an oral drug delivery system has been evaluated. First experiment designed to assess the *in vitro* biocompatibility of cationic branched polymers; poly(vinylamine)(vinylformamide) (PVF) copolymers and two different classes of dendrimers; diaminoethane (DAE) and polyamidoamine (PAMAM) dendrimers of different generation (Mw 289-50,865 Da) and different surface characteristics (-COONa, $-NH_2$). The haemolysis of PVF copolymers is increased as a function of amine content. Dendrimers with an amine surface were generally toxic depending on their concentration and generation. In contrast, dendrimers with carboxylate surface were shown to be biocompatible. Non-toxic PAMAM dendrimers were radioiodinated and used to investigate their ability to traverse rat intestinal tissue *in vitro*. The rate of serosal transfer and tissue uptake for anionic dendrimers was in the range of 3.4-4.4 and 0.6-2.5 μl/mg protein/h, respectively, whereas for cationic dendrimers these values were in the range of 2.3-2.7 and 3.3-4.8 μl/mg protein/h. After oral administration *in vivo*, most of the dose recovered was found in stomach and small intestine in 1 h, and after 5 h entered the caecum and colon. By 24 h most radioactivity (70-90% of the recovered dose) was located in faeces. The amount of radioactivity found in the blood was insignificant (0.1-1%). As 10-30% of the administered dose was not found in the organs studied the extent of oral absorption of dendrimer is so far inconclusive.

Finally studies were carried out using PAMAM dendrimers to investigate their ability to complex with piroxicam and indomethacin. Dendrimer gen 3 formed complexes with both drugs in aqueous solution. However, the interaction showed insufficient control of drug release to allow *in vivo* application. Therefore, a preliminary study was conducted to synthesis and characterise covalent drug-dendrimer conjugates using ibuprofen as a model drug. Overall the observations made in these studies show that dendrimers do have potential as drug carriers for oral delivery and in future dendrimers must be more specifically designed to promote oral absorption and subsequent controlled release.

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Abbreviations

DAB dendrimer	Diaminobutane dendrimer, Poly(propyleneimine) dendrimer
DAE dendrimer	Diaminoethane dendrimer
DDW	Double distilled water
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
EDA	Ethylenediamine
EDC	1-Ethyl-3(3-dimethylaminopropyl)carbodiimide
EI	Endocytic index
EPR effect	Enhanced permeability and retention effect
FCS	Foetal calf serum
GALT	Gut-associated lymphoid tissue
gen	Generation
GI	Gastrointestinal
GPC	Gel permeation chromatography
HMDS	Hexamethyldisilazane
HPMA	N-(2-hydroxypropyl)methacrylamide
HRP	Horseradish peroxidase
Mw	Molecular weight
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide
NSAIDS	Non-steroidal anti-inflammatory drugs
NVPMA	poly(N-vinylpyrrolidone-co-maleic acid)
PAMAM dendrimer	Polyamidoamine dendrimer
PBS	Phosphate buffer saline
PEI	Poly(ethyleneimine)
PP	Peyer's patches
PVF	Poly(vinylamine)(vinylformamide)
PVP	Poly(vinylpyrrolidone)
RBC	Red blood cell
SEM	Scanning electron microscopy
Sulfo-NHS	N-hydroxysulfosuccinamide

Chapter One

General Introduction

The oral route is the most popular route for drug administration because it offers convenience, patient compliance and low cost of healthcare. However, this conventional route of drug administration frequently provides insufficient oral bioavailability and poor control of drug concentration in the bloodstream or in the body tissues. The oral route is also unsuitable for administration of biotechnology products such as peptides, proteins and vaccines. Examples of therapeutically important drugs which are active parenterally but inactive or show only minor activity when administered orally are shown in Table 1.1. Poor oral bioavailability usually results from poor absorption and/or instability of drug in the gastrointestinal (GI) tract although drug metabolism in the GI tissues and liver also contribute to this problem.

Over recent decades numerous approaches have been explored with the aim of promoting oral absorption, improving drug stability in the GI tract and/or facilitating local GI delivery (Table 1.2). Strategies that are now well established include (1) Use of enteric coated dosage forms which protect drug from the acid pH of the stomach or gastric enzymes and release contents in the intestine or colon (Bechgaard & Christensen 1982) and (2) use of prodrugs e.g. sulphasalazine; 5-aminosalicylic acid and sulphapyridine linked by an azo bond and designed for activation in the colon thus being suitable for the treatment of inflammatory bowel disease (Ashford & Fell 1993). Although many novel oral drug delivery systems have been proposed and tested experimentally including liposomes, microparticles, nanoparticles, and water soluble polymers, so far none have been transfered to routine clinical use.

During the last decade the successful synthesis of novel symmetrical branched nanoscopic polymers known as dendrimers has opened the new opportunities in drug delivery (reviewed by Uhrich 1997, Bosman et al. 1999). The typical dendrimer structure is shown in Figure 1.1. A dendrimer consists of three architectural components: a core, layers of repeating units radially attached to the core, and an outer surface of terminal functional groups. The properties of dendrimers will be described later in section 1.5, but due to their unique structural properties it seems feasible that dendrimers may provide an opportunity to design novel oral delivery systems which have the potential to:

1) Increase solubility of poorly soluble drugs

2) Control the rate of release

3) Control GI transit of drugs by acting as new oral bioadhesive

4) Promote transfer across the GI epithelium

5) Target to specific regions in the GI tract and/or target to specific organs following oral absorption.

Table 1.1. Examples of drugs which have low oral bioavailability

Drugs	Problems	Route	References
Antibiotics			
Azithromycin	poor absorption	parenteral	Luke & Foulds 1997
Cephalosporin	poor absorption, metabolism	parenteral	Wu et al. 1995
Kanamycin	poor absorption	parenteral	Chien 1992
Penicillin G	poor absorption	parenteral	Chien 1992
Streptomycin	poor absorption	parenteral	Chien 1992
Anti-cancer drugs			
Taxol	poor absorption, metabolism	parenteral	Walle & Walle 1998
Antihistamines			
Disodium cromoglycate	poor absorption	parenteral	Chien et al. 1989
Antimycotic drugs			
Amphotericin B	poor absorption	parenteral	Montes 1996
Itraconazole	metabolism	parenteral	Luke & Foulds 1997
Antiviral drugs			
Acyclovir	poor absorbtion	oral, topical, parenteral	Fiddian 1995
Cardiovascular drugs			
Nitroglycerin	metabolism	parenteral sublingual transdermal topical	Heinzow & Ziegler 1981 Mar 1982
Propafenone	metabolism	parenteral	Monti & Saettone 1997
Verapamil	poor absorption	parenteral	Chien 1992
Central nervous system drugs			
Cocaine	metabolism	parenteral topical	Stewart et al. 1979
Hormones			
Insulin	poor absorption, metabolism	parenteral	Aungst 1988; Machida 1993
LHRH and analogs	poor absorption, metabolism	parenteral	Vickery 1985
TRH	poor absorption, metabolism	parenteral	Schurr 1985
Sex hormones			
Progesterone	poor absorption metabolism	Parenteral	Chien et al. 1989; McAuley et al. 1996
Testosterone	metabolism	Parenteral	Chien et al. 1989

Table 1.2. Methods for improvement of poor absorption and instability of drugs in the GI tract

Methods	Review references
I. Drug related approaches	
Change in physicochemical properties	Ungell 1993 Pauletti et al. 1997
II. Physiological related approaches	
Increase the membrane permeability	
-Absorption enhancers	Hochman & Artursson 1994, Wang 1996, LeCluyse & Sutton 1997
Prolong the residence time in GI tract	
-Use of bioadhesive polymers	Lehr 1992, Wang 1996 Ponchel & Irache 1998
-Heavy pellets, floating dosage forms, swelling dosage forms	Timmermans & Moes 1990 Moes 1993
Receptor targeting	Brayden & O'Mahony 1998
M cell targeting	Brayden & O'Mahony 1998
Stabilisation towards enzymic or pH degradation	
-Enzymic inhibitors	Wang 1996 Bernkop-Schnurch 1998
-Colon targeting	Ashford & Fell 1993, Rouge et al 1996
III. Dosage form-related approaches	
Stabilisation to enzymic degradation and/or target to specific organs	
-Enteric coated dosage forms	Healey 1990
-Lipidic vehicles (emulsions)	Wang 1996
-Liposomes	Devissaguet et al. 1990, Wang 1996
-Nanospheres, microspheres	Devissaguet et al. 1990, O'Hagan 1990, Kreuter 1996, Wang 1996, Alleman et al. 1998
-Cyclodextrins	Uekama et al. 1994

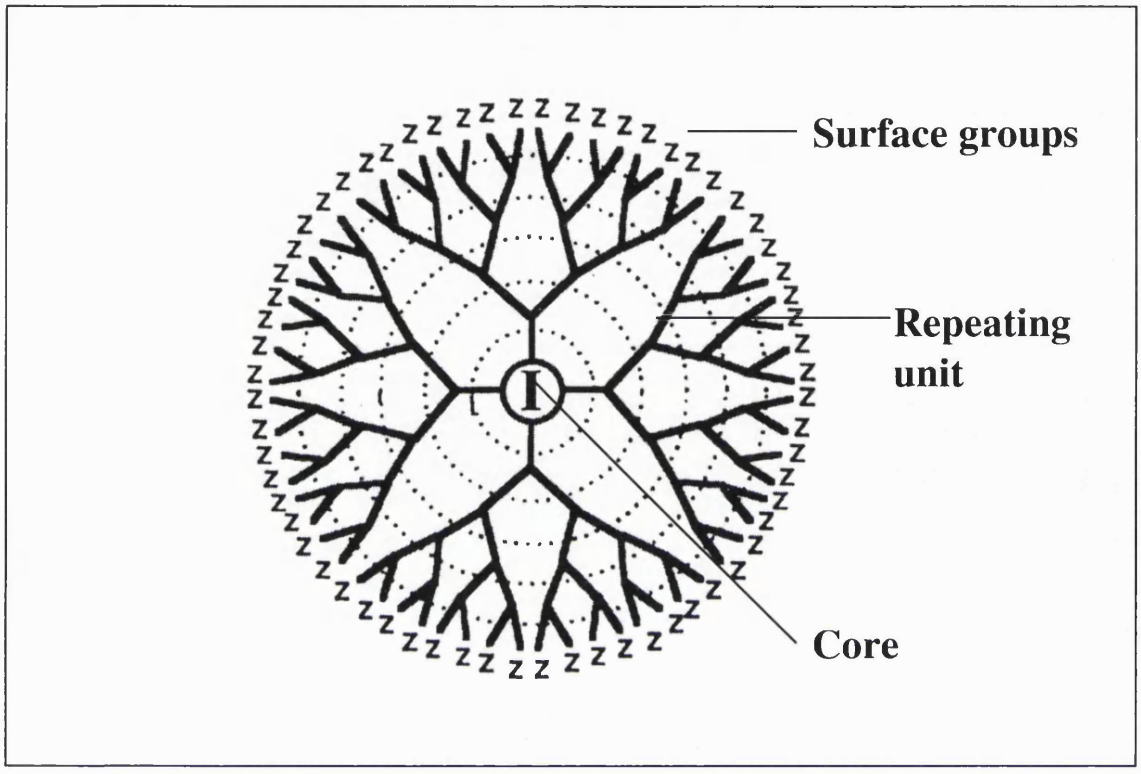


Figure 1.1. A typical structure of dendrimer (from Tomalia et al. 1990)- each dotted circle represents an increase of one generation (gen)

Therefore, in this study the potential of dendrimers acts as novel oral drug delivery systems has been studied systematically.

To understand the factors important for the rational design of dendrimer systems, it is important to understand the relevant background. For example general GI anatomy, the nature of the GI barriers that normally limit drug absorption and the other drug carrier systems that have been previously investigated for oral delivery. Also it is important to understand the basic chemistry and biological properties of dendrimers. These topics will be reviewed here.

1.1. The Anatomy of the GI tract

The GI tract is composed of three major compartments: the stomach, the small intestine and the large intestine (Figure 1.2a). When any oral drug delivery system is administered, the first compartment it will meet is the stomach. The stomach is an organ with a capacity for storage and mixing. Its lining consists of a large number of gastric pits which contribute to the storage capacity of the stomach and the stomach is divided into the cardia, fundus, corpus and pyrolic regions. There are two main secretions, mucus and gastric acid which are produced by the goblet cells and the parietal cells in the stomach lining (Chien 1992). Both mucus and gastric acid can influence the absorption and stability of drugs and delivery systems and their potential effect on dendrimers will be discussed later in section 1.2. The stomach is not generally involved in drug absorption due to its limited surface area (0.1 - 0.2 m^2), the lack of villi on the mucosal surface, a thick coating of mucus and also the short residence time of drugs in the stomach.

The principal sites of drug absorption are to be found in the small intestine. The small intestine is a tubular viscous organ approximately 5-6 m in length in humans and it has three main subdivisions: the duodenum, jejunum and ileum (Wilson et al. 1989). The intestinal epithelium contains many different cell types including goblet cells (secretion of mucus) and the columnar cells called enterocytes which are the major absorptive cells of the GI tract. Underlying the epithelium is a layer called lamina propia, which is supported by a layer of smooth muscle, the muscularis mucosa. These three layers-epithelium, lamina propia, and muscularis mucosa are collectively termed the intestinal mucosa (Figure 1.2b). The mucosa of the small intestine is folded into series of fingerlike projections known as intestinal villi, which have a height of 0.5-1.5 mm (Figure 1.2c), and it is the large number of villi present that create an enormous surface area extending to approximately 4,500 m^2. From the proximal to distal region of the small intestine the surface area progressively decreases. Each villus has a rich blood supply and also lymphatic vessels (lacteals) as shown in Figure 1.2c (Haeberlin & Friend 1992). The

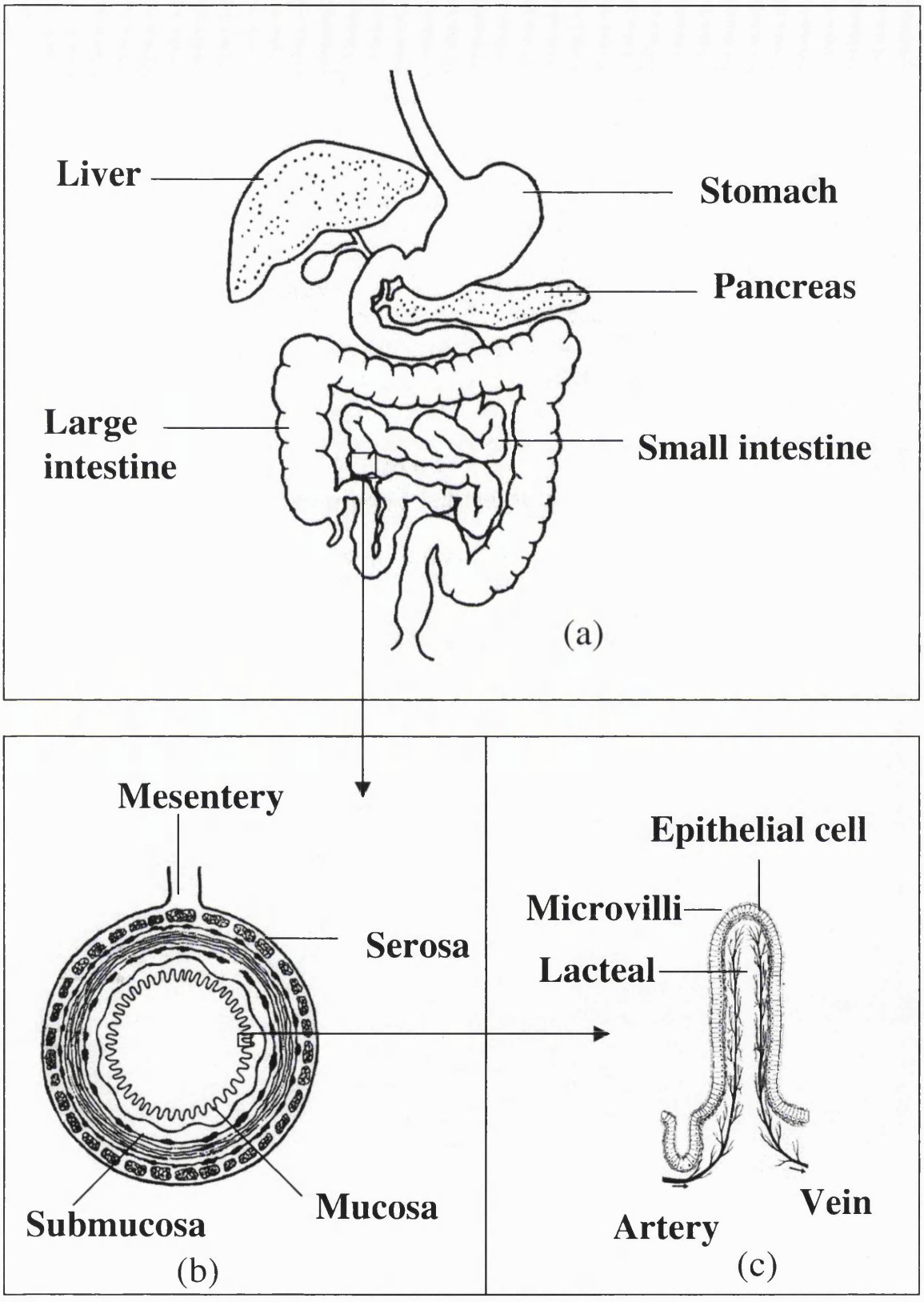


Figure 1.2. (a) Human gastrointestinal tract, (b) The cross-section of the intestine, (c) Intestinal villus (Adapted from Lamb et al. 1984; Haeberlin & Friend 1992)

enterocytes have an apical membrane containing many microvilli which again increase the apical surface area up to 14 to 40 fold. On the surface of microvilli, there is a filamentous coat which consists of sulfated mucopolysaccharides called the glycocalyx. The glycocalyx is in turn coated by a thick layer (up to 100 μm) of mucus. Both the mucus and glycocalyx offer a certain level of resistance to drug diffusion due to the viscosity of the network and the possible interaction between the drug and network components. The role of mucus as a barrier to drug absorption and a site for bioadhesive drug delivery system will be discussed later under section the GI barriers (section 1.2).

About 25 % of the mucosa surface is composed of the gut-associated lymphoid tissue (GALT). This is visible in the form of Peyer's patches (PP) which consist of aggregated lymphoid follicles in a rectangular or oval shape. PP are usually situated in the antimesenteric border of the intestine and extend through the intestinal mucosa (Haeberlin & Friend 1992), and they are most prominent in ileum where they are characterised by the presence of a small number of specialized absorptive epithelial cells called M (microfold) cells which are intermittently distributed among the major absorptive cells (Wang 1996). The major role of PP is the induction of a secretory immune response towards ingested antigen (Fasano 1998). Recent evidence suggests that M cells play an important role in the absorption of microparticles (reviewed by Yeh et al. 1998) and M cell-targeted microparticles hold promise for the delivery of vaccines. The absorption of particles via PP is reviewed in more detail in section 1.4.2.

The last anatomical compartment of the GI tract is the colon (or large intestine) which extends from the ileocecal junction to the rectum. It is composed of the cecum, the ascending segment, the transverse segment, the descending segment, and the sigmoid region. The total length of the colon is typically 1.5 m in humans (Mrsny 1992). Its principle functions are the reabsorption of water and the elimination of undigested materials. The colon lacks the extensive villi seen in the small intestine but contains variety of metabolic enzymes-producing bacteria. The most prevalent anaerobes are *Bacteroides sp.* and *Bifidobacterium* whilst the most numerous aerobes are *Eschelichia coli*, *Enterococci* and *Lactobacillus* (Steed et al. 1989). The presence of large number of bacteria concentrated in colon has stimulated interest in investigating carrier systems which are stable in the conditions existing in the stomach and the small intestine but which are degraded by the colonic bacterial enzymes. For example, use of azo bonds designed for clevage by bacterial enzymes have been used in prodrugs and azopolymer and polysaccharide coatings have been used in various dosage forms (reviewed by Ashford & Fell 1993). Colonic delivery has become a widely proposed route for the administration of poorly absorbed drugs and also it has been proposed as a site for peptide delivery due to the longer residence time, claims of low enzymatic activity, and

the suggestion of a greater responsiveness to agents that enhance the absorption of drugs compared to the upper GI tract (Mrsny 1992, Ashford & Fell 1993). It is envisaged that dendrimers may have potential for local delivery in the colon and this is discussed in Chapter 7 and 8.

1.2. GI Barriers to drug absorption

Whilst passing through the GI lumen a drug will be exposed to a number of adverse conditions before it can enter the bloodstream and also it must pass across the GI mucosa. The GI barriers to drug absorption can be divided into (1) the physical barriers and (2) the biochemical barriers as illustrated in Figure 1.3.

1.2.1. The physical barriers

Transit time

The transit time of a dosage form through the GI tract can be very variable. Sometimes it can be of very short duration (Rouge et al. 1996) and thus, the time available for the drug absorption might be limited. The average transit time in human is 24 to 72h (mouth to anus). The transit times through the stomach and small intestine vary less than the colonic transit times. The residence time of a solid dosage form in the stomach varies from a few minutes to several hours, depending on the feeding state and GI motility at the time of administration (Haeberlin & Friend 1990). Mrsny et al. (1993) reported typical residence times of approximately 60-90 min in the stomach, 30-40 min in the duodenum, 1.5-2 h in the jejunum, 5-7 h in the ileum and 35-36 h in the colon. For drugs whose absorption is limited to certain sites along the GI tract it is considered important to prolong their residence at the particular absorption site. For example, many approaches have been developed to increase the gastric residence time for drugs predominantly absorbed in the upper GI tract or for drugs that are poorly soluble in intestinal fluid such as diazepam, chlordiazepoxide and cinnarizine. Examples of these approaches are summarised in Table 1.2.

Bioadhesive polymers for example N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer (Bridges et al. 1988), polycarbophil (Lehr 1992), and bioadhesive protein tomato lectin (Naisbett & Woodley 1995) have been proposed as a means to slow down intestinal transit. In many cases, charged polymers showed better bioadhesive properties than the uncharged analogues (Bridges et al. 1988, Pato et al. 1994). Due to their numerous surface groups, which can become charged at physiological pH (van Duijvenbode et al. 1998), it may be possible for dendrimers to bind to surfaces electrostatically, and therefore prolong GI transit. Most bioadhesives tested to date are believed to interact with either the mucosal cell surface or, more frequently, with the

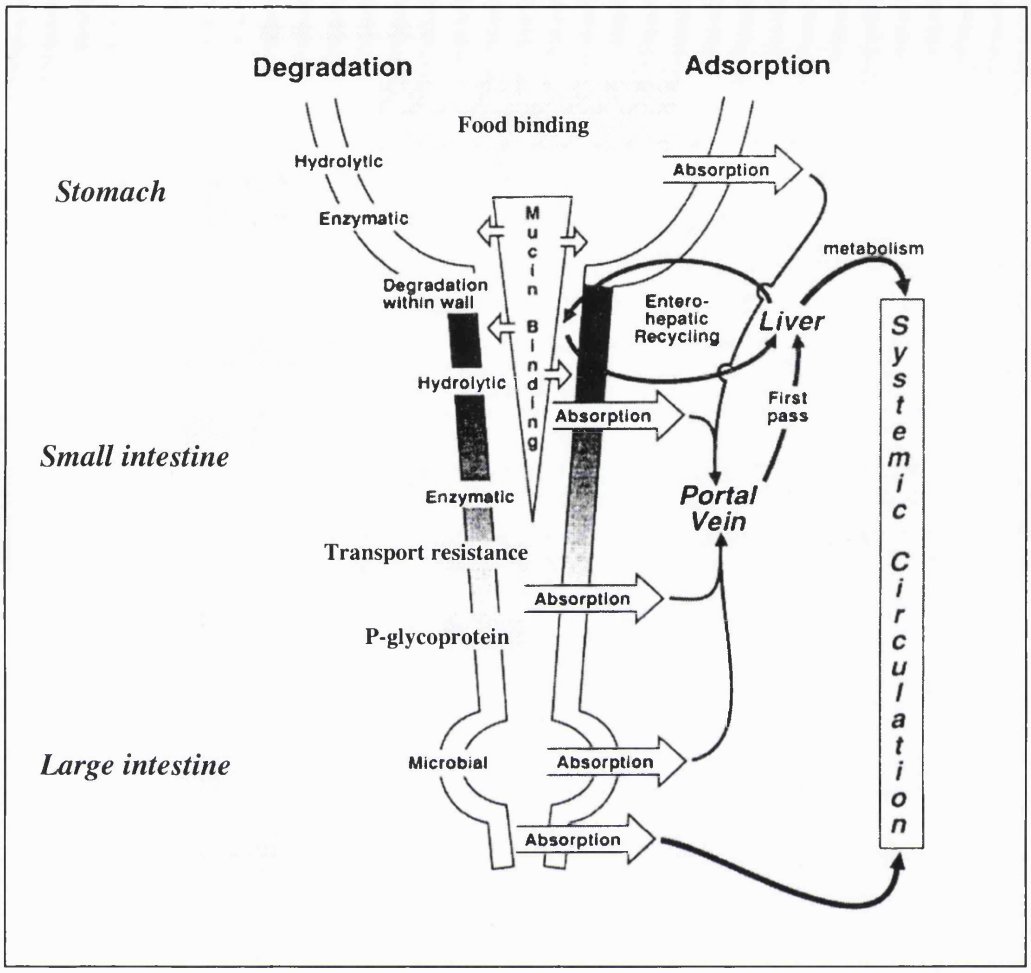


Figure 1.3. Barriers to the oral absorption of drugs (adapted from Chien 1992)

mucus present in the GI tract (Naisbett & Woodley 1994). Achieving long-term bioadhesion by interaction with mucus has been found to be limited due to the rapid turnover rate of mucin *in vivo* (Hayashi 1993). Specific interaction between polymers and the epithelial cell surface has been shown to achieve longer term bioadhesion compared to that of nonspecific interaction (Junginger 1993).

Mucus

The GI mucosa is coated with a layer of mucus that serves as a lubricant and protective barrier. The thickness of the mucus layer covering the tissue surface can vary from 5 to 500 μm (MacAdam 1993). Mucus consists of high molecular weigt (Mw) glycoproteins, termed mucin which are secreted by globlet cells and these are responsible for the cohesive and adhesive nature of the mucus layer. These glycoproteins consist of a protein backbone with oligosaccharide side-chains attached to specific amino acid residues. There are 160-200 oligosaccharide side-chains in each glycosylated region and the side chains usually contain five sugar residues: galactose, fucose, N-acetylgalactosamine, N-acetylglucosamine and N-acetylneuramic acid (also known as sialic acid). Fucose and sialic acid often occur as the terminal sugars and sulphate esters of galactose or N-acetylglucosamine are also frequently present. Since both sialic acid and sulphate esters of galactose or N-acetylglucosamine have pKa values of less than 3, such groups would be fully ionised at all but the lowest pHs in the GI tract, resulting in the mucus glycoprotein molecule carrying a negative charge. This charge may be partially responsible for the viscous property of the mucus gel as it would produce electrosatic repulsion between adjacent carbohydrate chains, resulting in expansion of the structure. Other constituents of the mucus layer include enzymes, electrolytes, water and sloughed off epithelial cells. Due to its viscosity, mucin acts as a diffusion barrier. Depending on the chemical nature of the drug administered in some cases the mucin may also bind the drug by electrostatic or hydrogen bonding. For example tetracycline hydrochloride (Kearney & Marriot 1987), some aminoglycoside antibiotics and β-lactam (Niibuchi et al. 1986) have been shown to bind to mucin.

Many studies have claimed to examine the influence of mucus layer on drug absorption but direct comparison between these studies is often complicated since different models have been used. The most commonly used *in vitro* drug absorption model is the Caco-2 cell monolayer system. Caco-2 cells do not secrete mucus. A mucus-producing human globet cell line, HT29-H cell line has been recently introduced as a screening method and this may give more meaningful insight into the role of mucin (Karlsson et al. 1993).

It is likely that only the diffusion of large molecules and particles is limited by the mucin coating as small molecules pass easily due to high water content of mucus (Wilson et al. 1989; Squier 1992). Dendrimers are very small macromolecules in nanometer size range (~1-10 nm) (Tomalia el al. 1990) and depending on the dendrimer surface groups present they can be charged at physiological pH. Dendrimers might be expected to diffuse through the mucus layer to reach the cell surface for further absorption. However, there is also the possibility that dendrimers might interact electrostatically with the negatively charged mucin glycoprotein and act as bioadhesive drug delivery systems.

Hydrophobic membranes and tight junctions

The cell monolayer of the intestine is composed of two parallel barriers: the epithelial cells and the tight junctions. The drug absorption route through the epithelial cells is called the transcellular route, while the parallel route through the tight junction between the cells is called the paracellular route. The mechanisms of drug absorption will be described later in section 1.3. Chemically, the cell membrane consists of a lipid bilayer matrix with a variety of integral and peripheral proteins. The proteins contribute to the structural integrity of the cell as well as its transport and metabolic capabilities. In contrast, the nature of the lipids influences both the fluidity of the cell membrane and also anchors the protein structure to the core of the membrane via specific protein-lipid interactions (Pauletti et al. 1997). The ability of a drug molecule to partition into the cell membrane is of critical importance in determining its ability to permeate the intestinal membrane via transcellular pathway. As the cell membrane is considered a lipophilic barrier, the lipophilicity of drug molecule has been viewed as the single most important molecular characteristic determining passive diffusion through membranes. However, the results from *in vivo* experiments suggested that an "optimum" lipophilicity improves permeation rather than a "high" lipophilicity. In addition, other physicochemical properties of a drug molecule e.g. size, charge, molecular conformation can also influence absorption (Conradi et al. 1991; Artursson 1993).

The mucosal epithelial cells are attached to one another by a complex, apical, junctional complex. There are three types of cell junctions: tight junctions (zonula occluden), gap junctions and desmosomes (zonula adherens) (Wilson et al. 1989). There is now substantial evidence that the intestinal tight junction plays a pivotal role in epithelial permeability. Tight junctions are formed when specific proteins in two adjacent interacting plasma membranes make direct contact across the intercellular space. These can be visualized as linear strands of intermembrane proteins. A belt-like structure composed of many protein strands completely encircles each cell in the sheet, attaching it to its neighbours. Within these strands are pores which have limited permeability to small hydrophilic molecules. The calculated effective pore radii of the human small intestinal

epithelium are about and 7-9 Å for duodenum and jejunum and 3-5 Å for ileum (Artursson 1991; Madara 1991; Hillgren et al. 1995). They have been reported to be impermeable to molecules with radii larger than 11-15 Å (Madara et al. 1985; Burton et al. 1987; Ho et al. 1990). As dendrimers are nanometer-sized macromolecules, it is unlikely that they would be transported across the intestine via these intercellular tight junctions unless they are very small (e.g. 1-2 nm) or alternatively the dendrimer can open the tight junctions. The mechanism of absorption of dendrimers across the GI tract will be discussed later in section 1.3 and in Chapter 4.

1.2.2. The biochemical barriers

pH

The pH of the luminal contents throughout the GI tract is of great relevance to drug delivery since approximately 75% of all clinically utilised drugs can behave as either weak acids or bases (Hollinger 1997). The intraluminal pH can influence the solubility and the degree of ionisation of a drug, with predictable effects on the rate of absorption. The pH ranges from 1-3 in the stomach, it rapidly rises to a pH of > 5 in the duodenum and rises again to a pH of 6.6-7.5 in the jejunum and ileum (Haeberlin & Friend 1990). On the basis of the pH-partition hypothesis, the absorption of acidic compounds (pKa < 4) should be optimal in the stomach, and the absorption of basic compounds (pKa > 6) should occur in the small intestine. However, in the small intestine drugs frequently have longer transit time and the villi and microvilli provide a very large membrane surface area for absorption so the absorption of most weakly acidic and neutral compounds takes place largely in the small intestine (Hollinger 1997).

The pH in the GI tract would also be expected to influence the degree of ionisation and thus charge characteristics of dendrimers. Dendrimers contain a large number of both interior and exterior groups that can show different degrees of protonation at different pH (van Duijvenbode et al. 1998). The effect of dendrimer charge on absorption across the GI tract and the ability to form stable dendrimer complexes with drugs will be discussed later in chapter 4 and 6, respectively. The influence of the pH on the extent of drug absorption (Oberle & Amidon 1987; Lee et al. 1994) as well as on the degradation of drugs has been widely studied (Aungst 1993; Langguth et al. 1994). Examples of compounds that are unstable at the stomach pH include some penicillins, omeprazole and peptide drugs. In many cases this problem has been easily overcome using enteric-coated drug particles, or by the formulation with antacids (Hartman et al. 1991).

Enzymes

In a positive sense the enzyme systems present in the intestine do have the ability to transform pharmacological inactive drugs into their active parent compounds. However, many of the GI enzymes, have a detrimental as they prematurely metabolise many drug substances. The enterocytes contain diverse metabolic activities including the most important class of phase I metabolic enzymes (i.e. cytochrome P-450s) (Peters & Kremers 1989; Watkins 1992), several phase II conjugation enzymes (Peters et al. 1989), and also esterases (Whitaker 1994) and peptidases (Erickson 1992). Because of the abundance and variety of proteases in the GI tract, the enzymatic barrier is considered the most important in limiting the absorption of protein drugs. Hydrolysis of peptides and proteins can occur luminally, at the brush border and intracellularly (Woodley 1994). Using horseradish peroxidase (HRP) to study transport in rabbit jejunum *in vitro*, only 3% of the total HRP transport from mucosal to the serosal surface was found to be intact HRP (Heyman et al. 1982). In another study, insulin was found stable for 30 min when present in the incubation medium used for everted rat intestinal sacs cultured *in vitro*, but it was almost completely degraded by homogenates of the duodenum or jejunum within the same period of time (Schilling & Mitra 1990). A later report confirmed that 92% of the total insulin-degrading enzyme activity was found in the cytosol of the rat small intestine (Bai & Chang 1995). However, it is probable that the cytosolic extracts contained lysosomal enzymes. Another source of enzymes in the intestinal lumen is the presence of intestinal cells that have been sloughed off from the villi and shed into the lumen. These cells contain both intracellular and brush-border enzymes (Woodley 1994). A further source of degradative enzymes are bacteria, particularly in the large intestine (Faigle 1993).

Much effort has been made to protect drug molecules from these degradative enzymes (Table 1.2). Specific inhibitors can be employed to stabilise protein or peptide (reviewed by Bernkop-Schurch 1998). A number of devices (Prisant et al. 1992), including micro- or nano-particles (Damge et al. 1988; O'Hagan 1990) and lipid formulations (Ho et al. 1996) have been used to limit the extent of catabolism by physically sequestering drugs thus protecting them from enzyme activity. Although as yet there have been no systematic studies investigating the degradation of dendrimers, by pH or by enzymes commonly present in the biological environment, many of the synthetic structures described are probably non-biodegradable e.g. polyamidoamine (PAMAM) dendrimers. It is interesting to note that some types of dendrimers (Jansen et al. 1994) provide an interior structures that might allow encapsulation of the drugs and it could be expected that such dendrimers might protect a drug against degradation in the GI environment.

p-Glycoprotein

P-glycoprotein is a 170-180 kDa membrane glycoprotein that acts as an ATP-dependent efflux pump, thereby reducing the intracellular accumulation or transcellular flux of a wide variety of drugs. Although these efflux system are most commonly observed in tumor cells, they are also present in a variety of normal tissues including liver, brain, kidney and the GI tract (Thiebault et al. 1987). P-glycoprotein is located on the apical membrane of intestinal epithelium and acts as an efflux pump that transports drug molecules out of mucosal epithelial cells back into the lumen as they are absorbed across the intestinal mucosa (Hebert 1997; Watkins 1997). In recent years, it has been realized that p-glycoprotein plays an important role in limiting oral absorption of drugs, including cyclosporin (Augustijns et al. 1993), certain peptides (Augustijns et al. 1993; Aungst & Saitoh 1996), digoxin (Tanikawara et al. 1992), fluoroquinolones (Griffiths et al. 1994), ranitidine (Cook & Hirst 1994) and β-adrenoceptor antagonists (Karlsson et al. 1993). Verapamil and quinidine are drugs that are known to inhibit p-glycoprotein (Ford & Hait 1990). Various methods have been used to study the role of p-glycoprotein in the GI tract and these include Caco-2 cells (Hunter et al. 1993; Cavet et al. 1996), everted gut sac methods (Leu & Huang 1995; Barth et al. 1998) and *in situ* perfusion (Leu & Huang 1995).

It is important to know whether poor absorption of any drug is simply due to the presence of a secretory transport system. Many are investigating whether drugs that inhibit or disrupt intestinal p-glycoprotein can be used to increase absorption of drugs resulting in increased oral bioavailability (Leu & Huang 1995; Sparreboom et al. 1997).

1.3. Mechanisms of absorption

Theoretically, two major pathways are available for the passage of molecules from the intestinal lumen to the bloodstream; transcellular and paracellular (Figure 1.4). The transcellular pathway includes several mechanisms of drug transfer: passive diffusion, passive carrier-mediated transport, active carrier-mediated transport, and endocytosis. The characteristics of each transport mechanism are summarised in Table 1.3.

1.3.1. Transcellular pathway

Passive diffusion

Passive diffusion is mostly used by the small lipophilic molecules or low Mw drugs which are generally rapidly absorbed (Narawane & Lee 1994). When using this pathway, the drug molecule undergoes a series of partitioning and diffusion processes prior to entry into the systemic circulation. Partitioning occurs between the aqueous compartments (the unstirred water layer, mucus, cytoplasm and blood) and hydrophobic

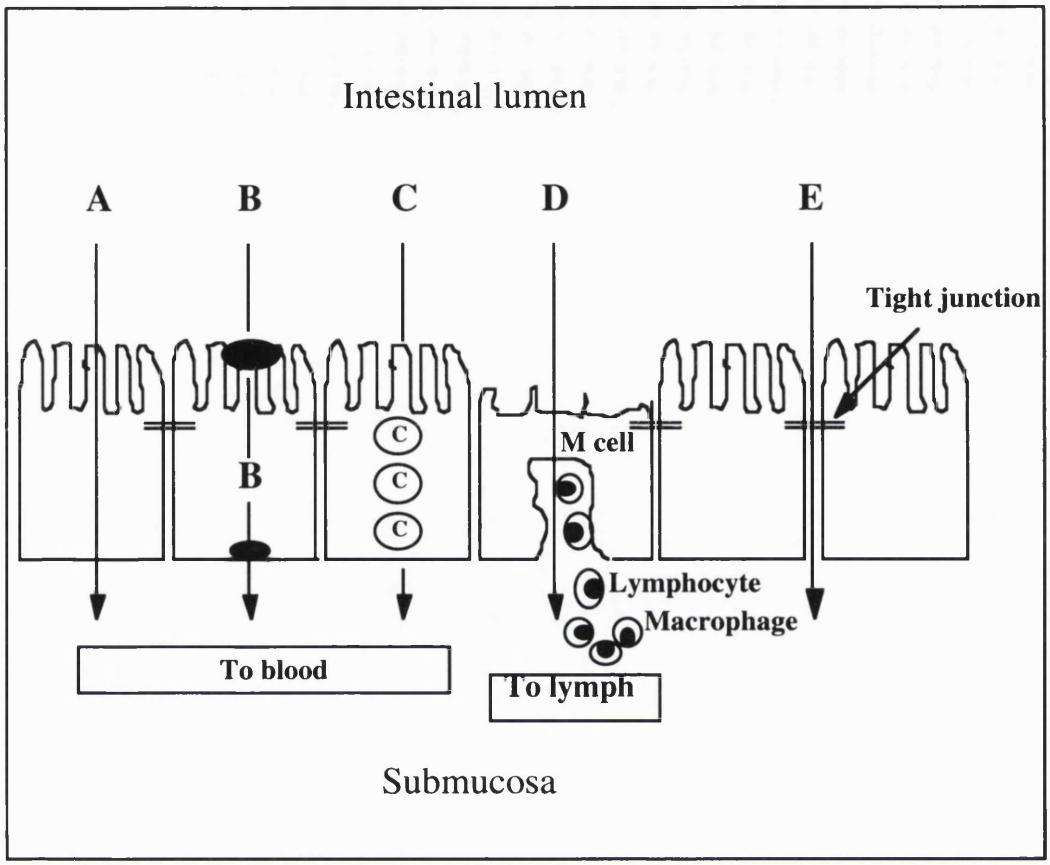


Figure 1.4. Pathway of intestinal absorpion. Transcellular route (A-D); (A) passive diffusion, (B) carrier mediated transport, (C) endocytosis, (D) transport of particles via M cell, and Paracellular route (E)

Table 1.3. Characteristics of transport mechanisms

Mechanism	Characteristics
Transcellular	
Passive diffusion	depends on the concentration gradient
Passive carrier-mediated	carrier-mediated saturable with increasing concentration competitive substrates
Active carrier-mediated	carrier-mediated can be against concentration gradient energy dependent inhibition by metabolic inhibitors substrate competitive, saturation
Endocytosis	usually energy-dependent can be against concentration gradient inhibition by metabolic inhibitors
Fluid-phase	soluble molecules are internalized with the vesicle volume non-specific and non-saturable
non-specific adsorptive	solute non-specifically adsorbs to cell surface proteins or glycolipids can be saturable and competitive often high capacity
specific adsorptive	substrate-specific high affinity and saturable usually low capacity
Paracellular	molecules cross tight junction and pass between the cell depend on concentration gradient

compartments (the membrane of the enterocyte and blood capillaries). Diffusion of the drug depends upon the magnitude of the concentration gradient, the membrane to be traversed, and then physicochemical properties of the drug such as pKa, partition coefficient and lipophilicity (Helliwell 1993).

Passive carrier-mediated transport

Passive carrier-mediated transport is based on many of the same principles that control passive diffusion, except that the drug molecule is transported by a carrier protein within the membrane which has selective binding site for hydrophilic substrates (Helliwell 1993). Drugs absorbed via this route, include, β-lactam antibiotics (Sanches-Pico et al. 1989; Hildago et al. 1995), cephalosporins (Gochoco et al. 1994) and ACE inhibitors (Friedman & Amidon 1990). These drugs are absorbed by amino acid and dipeptide transporters which are located in the apical cell membrane.

Active carrier-mediated transport

Active carrier-mediated transport involves the absorption of molecules via a specific membrane carrier and also requires the expenditure of metabolic energy. Absorption can occur against a concentration gradient. This pathway has been well documented as a membrane of transport for bile salts (Simon 1990), sugars (Tsuji 1986), amino acids, methotrexate (Said & Redha 1987) and some of the B class vitamins.

Endocytosis

Macromolecules can also be transported across mucosal epithelial cells via a vesicular transport pathway which is called endocytosis. Generally, endocytic phenomena fall into two broad categories, phagocytosis and pinocytosis. Phagocytosis can be defined as internalisation of particles larger than 500 nm (Holmberg et al. 1987; Eldem & Speiser 1989), whereas the pinocytosis has been defined as the engulfment of small droplets of extracellular fluid "cell drinking" (Pratten et al. 1980). The upper size limit for particle uptake across the enterocytes reported by Jani et al. (1992a) was about 100 nm.

During endocytosis, substrates may be captured in two possible ways, either taken up in solution (fluid-phase endocytosis) or following attachment to the invaginating plasma membrane by specific or non-specific adsorption (adsorptive endocytosis). Therefore substrates with no affinity for the membrane are taken up solely in solution, whereas substrates with membrane affinity can be taken up either membrane-bound, or by a combination of the fluid-phase and adsorptive pinocytotic modes (Pratten et al. 1980). The endocytic uptake mechanism requires energy and the process can be inhibited using inhibitors of both glycolysis and oxidative phosphorylation.

Enveloped viruses are usually taken into cells by membrane fusion while non-enveloped virus use endocytic mechanisms for cellular entry (Klasse et al. 1998). As dendrimers are of similar size as many viruses (Tomalia 1995) that they may be absorbed via the endocytic pathway. Although, it was originally believed that the major site of transfer of particles and microorganisms across the GI was the M cells of PP, later work has shown that there was no discrimination between enterocytes and M cells regarding uptake of small particles in the nanometer size range (Mathiowitz et al. 1997). Endocytic uptake and transfer across the GI (transcytosis) is not only dependant on the size of molecule transferred but also on other factors such as its shape, architecture, charge and hydrophobicity. The possible mechanisms of dendrimer transfer across the GI is discussed in Chapter 4.

1.3.2. Paracellular transport

Some hydrophilic and charged drugs can be passively transferred across the GI mucosa through the water-filled channels between epithelial cells and this route has been called paracellular route. These intercellular spaces represent only 0.01% of the total intestinal surface area and only very small molecules can pass through the tight junctions. Drugs which used paracellular pathway to cross the epithelial cell layer include hydrochlorothaizide (Taylor et al. 1989), small peptides (Rojanasakul et al. 1992; Adson et al. 1994; Bohner et al. 1995), and some nucleoside analogues (Sinko et al. 1994).

Various studies showed that intestinal permeability is not simply dependant on the Mw or molecular radius of a drug molecule but dependant on their molecular structure. In particular, the charge of drug molecule influences its absorption. Some studies (Rojanasakul et al. 1992; Adson et al. 1994; Pauletti et al. 1997) reported that cations are more easily transported through the tight junctions than are nonionic species or anions. In contrast other studies have suggested that anions are able to permeate by this pathway most efficiently (Rubas et al. 1994). Recently, it was found that enhancers such as calcium chelators or surfactants, could widen the tight junctions leading to increased drug absorption (Hochman & Artursson 1994; Knipp et al. 1997; Lutz & Siahaan 1997).

1.4. Colloidal carrier systems for oral delivery

Before discussing dendrimers as potential oral drug carriers it is important to briefly review other drug carrier systems that have been used as carriers for oral delivery. In recent years several type of colloidal carrier systems have been employed for oral delivery including liposomes, niosomes, nano- or micro-spheres and nanocapsules. These drug carrier systems have been used to localise drug at the target site (e.g. colon

delivery) and to allow sustained release of the drug when required. Drug carriers designed for oral delivery are usually expected to protect drugs from luminal degradation and in certain cases to promote transfer them from the mucosal to the serosal side of the GI barrier (Devissaguet 1992). In addition, drug-loaded carriers are proposed as a mean to avoid intracellular degradation metabolism and, once on the serosal side, allow drug release into blood or lymph. Here the use of liposomes, nanospheres and microspheres as oral drug carriers including their mechanisms of GI uptake will be briefly reviewed.

1.4.1. Liposomes

Liposomes have been used to encapsulate many drugs for oral delivery. This topic has been reviewed elsewhere (Woodley 1985; Weiner & Chieng 1988; Kimura 1989). Liposomes consist of one or more concentric spheres of lipid bilayer(s) surrounding an internal aqueous compartment(s). Their design allows hydrophilic drugs to be encapsulated in the aqueous core and hydrophobic drugs to be incorporated within the phospholipid bilayer. Liposomes were first proposed for oral delivery as they were found to protect the entrapped drug from digestive degradation (Bangham et al. 1965). Sessa and Weissman (1970) suggested that liposomes had the ability to increase oral drug absorption. Among all drugs tested in liposomes, insulin has been one of the most widely investigated. Although orally administered liposomal insulin has been claimed to penetrate the GI mucosa and exert a hypoglycemic effect, the results described so far have been extremely variable and contencious (Reviewed by Devissaguet 1992). Several studies have clearly demonstrated that liposome delivery systems are not suitable for use as oral drug carriers. They have been shown to be unstable in the presence of bile salts and enzymes in the GI tract. Even when *in vitro* techniques were used to study liposomes across intestinal tissue avoiding exposure to bile salts and enzymes, still no transfer of intact liposomes was observed (Patel et al. 1985; Weiner & Chieng 1988).

Many new approches have been used to try improve liposomal stability and promote their absorption. Surface coating with polyethylene glycol or the sugar chain portion of mucin produced an increase in liposomal stability in the GI tract (Iwanaga et al. 1997). Polymerized liposomes, prepared using azobis(isobutylronitrile) and azobis(2-amidinopropane) hydrochloride as radical initiators, showed increased stability when exposed to triton X-100 and were found to be trnsfer intact across the intestine (Okada et al. 1995). Liposomes have been proposed as an oral vaccine delivery system since they were absorbed via PP to lymphatic system (Harokopakis et al. 1994) and lectin modified liposomes were found to show improved to PP targeting (Chen et al. 1996).

1.4.2. Nanospheres and Microspheres

Nanospheres and microspheres are solid spherical matrix in the nanometer and micrometer size range, respectively. They are usually prepared from biodegradable polymeric materials. Drug incorporation to nano- and micospheres can be either by surface adsorption or by embedding within the matrix. Release of drugs occurs by bioerosion or diffusion through the matrix. Nano- and micro-spheres, even if biodegradable, are more stable than liposomes in the presence of an acidic pH, bile salts and enzymes. They can protect labile drugs from degradation in the GI tract as long as bioerosion does not occur (Devissaguet et al. 1992). In addition, drug release is related to the rate of carrier degradation rate allowing the possibility to develop a controlled release system (Tomlinson 1987).

Nano- and micro-spheres have been shown to improve oral delivery of many drugs (Maincent et al. 1986; Beck et al. 1994; Kreuter, 1994). A significantly prolonged reduction in the blood glucose levels of diabetic rats was obtained following the oral administration of insulin-loaded nanospheres (Damge et al. 1988; Kreuter 1994). The systemic bioavailability of vincamine-loaded nanospheres was increased 350% compared with an aqueous solution of vincamine (Maincent et al. 1986).

Numerous papers over the last two decades have demonstrated that particle uptake by the GI tract is a reality (Le Fevre et al. 1989; Ebel 1990; Eldrige et al. 1990; Jani et al. 1989; Jani et al. 1990; Jani et al. 1992a; Jani et al. 1992b; Florence et al. 1995; Hodge et al. 1995). Mechanisms proposed for the translocation include transcytosis, passage via the paracellular pathway and also uptake via the M-cells of the PP. The extent of oral absorption of nano- and micro-spheres seems to depend strongly on particle size and an upper size limit for transfer seems to exist. Uptake has been shown to increase with decreasing particle size (Jani et al. 1989; Jani et al. 1990; Kreuter 1994a) and increasing particle hydrophobicity (Eldridge et al. 1990). Generally, only very small particles between 20-50 nm can use endocytosis pathway in special cases maybe up to 200 nm (Devissaguet et al. 1992) and the passage of particles by the paracellular route seems rather rare unless the tight junction has been disrupted. Particle uptake and absorption via M cells and PP is still considered to be the most important pathway for uptake but particles > 5 μm in diameter have been shown to become trapped within the PP, whereas smaller particles can be orally absorbed through the efferent lymphatics (Eldridge et al. 1990). Due to the potential for uptake via PP, nano- and micro-spheres have received particular attention in relation to vaccine delivery via the oral route (Cox & Taubman, 1984; Eldridge et al. 1990).

One of the new approaches for improving the efficiency of oral delivery has been addition of bioadhesive substances to the nano- or micro-spheres. The carbopol-blended microspheres show prolonged 114% GI transit time (Akiyama et al. 1995). Tomato lectin has been used as a bioadhesive agent and it has been shown to enhance intestinal transcytosis of 500 nm polystyrene nanoparticles (Hussain et al. 1997). Mathiowitz et al. (1997) have shown that microspheres made of biological erodable polymer with strong adhesive property increase the absorption of three model drugs of different molecular size: dicumarol, insulin, and plasmid DNA.

1.5. Dendrimers as a potential oral delivery system

Dendrimers are macromolecules which contain well-defined highly branched structure and a large number of reactive chain-ends. The term dendrimer is derived from 'dendron' the Greek word for tree and 'meros' which means the smallest repeating structural unit (Tomalia et al. 1990). These branched macromolecules are also known by other names, such as arborol, cascade molecules or starburst polymers. By synthesis through a stepwise repetitive reaction sequence, dendrimers have well defined branched structure radiating from a central core. They have a specified size, shape and Mw depending on the generation (gen) (Tomalia 1995; Dagani 1996). Although other hyperbranched polymers e.g. some polysaccharides such as amylopectin, glycogen, and some other biopolymers are also highly branched their structure, this is neither regular nor highly symmetrical as found in the dendrimer and they also have a lower degree of branching (Frechet et al. 1994; Frechet 1996).

1.5.1. Synthetic approaches

Two major synthetic approaches have been used to prepare dendrimers: the divergent method and the convergent method (Figure 1.5).

Divergent method

The divergent method was originally developed by the groups of Denkewater (1983), Tomalia (1985), and Newkome (1985). Tomalia achieved the first successful synthesis of ideal highly branched PAMAM dendrimers through the 10th gen with a defined number of surface groups. In the divergent approach, synthesis is initiated at the center or core and proceeds outward to the surface. The method involves repetitive addition of a repeat branch unit and radiating out from the core and as more branches are subsequently added, the dendrimer is forced into a globular shape. Each addition of the branch unit referred to as a "generation" (Figure 1.1), essentially doubles the number of surface groups and Mw (Tomalia et al. 1990).

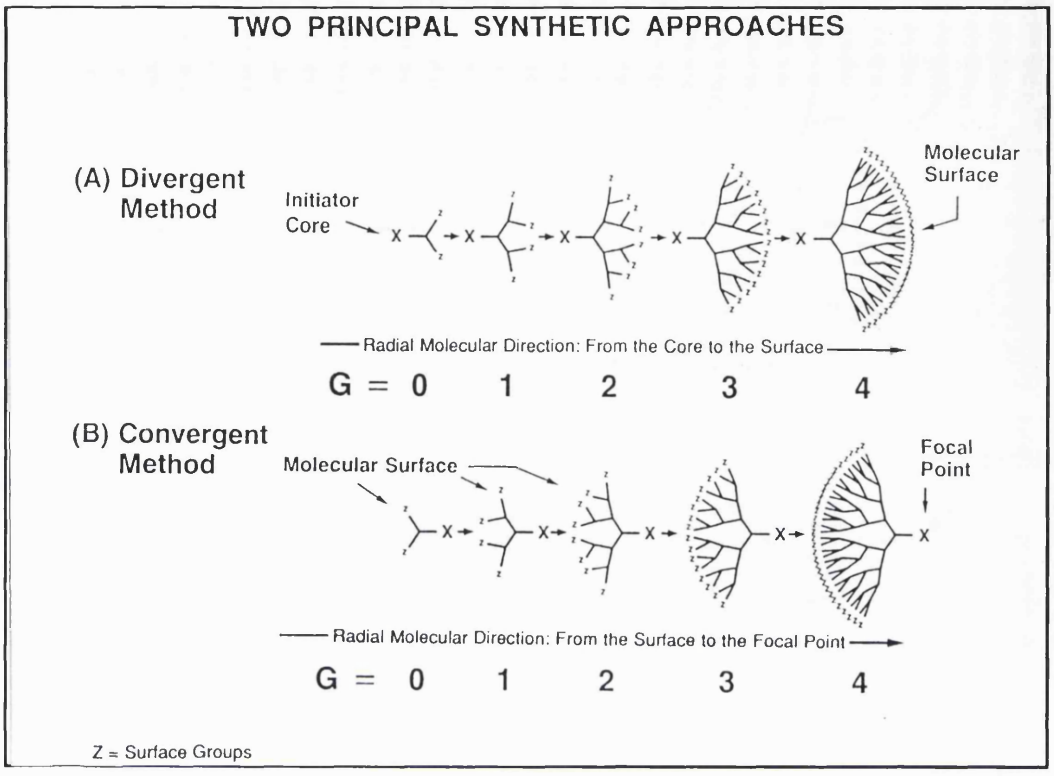


Figure 1.5. Synthetic approaches used to prepare dendrimers

A wide variety of dendrimer families have been synthesized using this method and the synthesis of high Mw dendrimers (>100,000 Da) in large quantity has been described. There are currently three families of dendrimer in commercial production: PAMAM dendrimers, poly(propyleneimine) dendrimers and hydroxylated polyester dendrimers (O'Sullivan 1993; Tomalia 1993; Tomalia & Durst 1993; Lorenz et al. 1995). However, because the number of chain ends increases rapidly at each stage of the divergent growth process, care must be taken to ensure that all reactive sites react in the same way in order to control the ultimate regularity of the dendrimers and avoid the introduction of side reactions causing imperfections.

Convergent method

The convergent process was introduced by Hawker & Frechet (1990) and Miller & Neenan (1990). This type of synthesis, in contrast to divergent method, starts at the surface and progress towards the core. Growth is begun at the chain ends and repetition of a two step-growth process involving coupling to the branching sites of the monomer and activate the single remaining reactive site. After the synthesis of "wedge" (large dendrons), these are finally attached to a polyfunctional core. The convergent method allows better control of the reaction stoichiometry and it is easier to purify the product at each step and to characterise the product (Frechet et al. 1994). Using the convergent method, it is possible to prepare segmented-block dendrimers or two- or three layers structures. However, synthesis of high Mw dendrimers is a limited due to steric problems involved at the final step when dendrons must be added to the core (Liao & Moss 1996). A comparison of the advantages and disadvantages of the divergent and convergent methods of synthesis is given in Table 1.4.

1.5.2. Physical properties of dendrimers

The dendritic architecture brings many new properties to the field of polymeric materials. In terms of architecture, the linear polymers have been compared with "cooked spaghetti" whereas dendrimers have been compared with "green peas" (Frechet et al. 1994). Dendrimers offer precise and systematic molecular sizes in the nanoscopic range (1-10 nm) and they provide shapes ranging from spheroids to ellipsoids to rods (Tomalia 1990; Yin et al. 1998).

Dendrimers can have high solubility in various solvents when compared to analogous linear polymers. Both their shape and functionality has a great effect on their solubility. By changing the chemical nature of the surface groups, dendrimers can be transformed from organic solvent-soluble polymers to water-soluble materials (Hawker et

Table 1.4. Comparison of the advantages and disadvantages of the divergent and convergent methods of synthesis (Ardoin & Astruc 1995; Liao & Moss 1996)

Advantages	Disadvantages
Divergent method	
1. Suitable for preparation of high molecular weight dendrimers (>100 000)	1. Excess reagent is needed
2. Less reaction sequences involved	2. Elimination of excess is required
3. Large quantity production is possible	3. Less control of product purity
Convergent method	
1. Block copolymers are possible	1. Molecular weight of dendrimer is limited due to steric problems
2. Reaction stoichiometry is under control	2. A more elaborate reaction sequence involved

al. 1993). Both hyperbranched polymers and dendrimers have significantly lower viscosities than linear polymers of the same Mw (Tomalia et al. 1990). The specific viscosity of dendrimer solutions goes through a maximum as a function of the Mw, while for linear polymers viscosity increases in direct proportion to Mw. Within a homologous series as the gen number increases, the molecule undergoes a transition from an extended to a globular shape to give molecules with a more compact, spherical shape (Tomalia et al. 1993; Frechet et al. 1994).

Studies using fluorescence probes and computer-assisted molecular modeling have indicated that the lower gen PAMAM dendrimers (gen < 3.5 or 4) have a more dome-like shape than the higher gens, which are believed to be more spherical (Tomalia et al. 1990). Dendrimers with a spherical shape possess well-developed internal hollows and channels. Determination of surface packing of the PAMAM dendrimer surface groups by viscosity and refractive index indicated a reduced interaction between the surface groups and the solvent below gen 4 (Nayler et al. 1989).

1.5.3. Biological properties of dendrimers

Although there has been an exponential increase in the number of publications in the dendrimer field in the last ten years, the number of studies related to their biological properties are still very limited. Tomalia (1990) was the first to point out that dendrimer properties, such as their dimensions, surface area, morphology and topology mimic the properties of important proteins and bio-assemblies. For example the PAMAM dendrimers of gen 3, 4 and 5 are roughly the same size and shape as insulin (~ 30 Å), cytochrome C (~ 40 Å) and haemoglobin (~ 55 Å), respectively. Cationic PAMAM dendrimers of gen 7-10 have also been proposed as a means to mimic histone for DNA binding. Examples of researches on dendrimers for drug and gene delivery are given in Table 1.5.

Glycodendrimers

The specific interactions between carbohydrates and proteins on cell surfaces are basis of many cell-adhesion phenomena. As these interactions are rather weak, synthesis of dendrimers with numerous sugar residues exposed on the surface was proposed as a means to increase the affinity. These "glycodendrimers" are finding increasing use as mimics of multifunctional glycoproteins. Sialic acid terminated dendrimers showed enhanced ability to bind and inhibit influenza A virus attachment to human erythrocytes. Glycodendrimers were approximately 10^6 times better than monosialosides in this inhibition assay (Roy et al. 1993; Roy 1996). A hexavalent spheroid dendrimer ending with D-mannopyranoside residues has also shown the ability to inhibit the binding of

Table 1.5. Examples of research on dendrimers for drug and gene delivery

Characteristics of research	References
Biocompatibility	
Cytotoxicity & biodistribution	Roberts et al. 1996, Kobayashi et al. 1999, Malik et al. 1999
Biodegradation	Seebach et al. 1996
Complexation/ Encapsulation of drugs	
Encapsulation of guest molecules	Jansen et al. 1995, Liu & Uhrich 1997
Complexation/conjugation with drug	Naylor et al. 1989, Wallimann et al. 1996, Esfand 1997, Malik 1999
Glycodendrimers	Roy et al. 1993, Lindhorst & Kieburg 1996, Page et al. 1996, Aoi et al. 1997, Thompson & Schengrund 1997
Peptide-based dendrimers	Nadelli 1992, Rao & Tam 1994, Shao & Tam 1995
Targeting anticancer agents	Malik 1999
Gene delivery	Belinska et al. 1996, Delong et al. 1997, Tang & Szoka 1997, Qin et al. 1998, Reuter et al. 1999
Imaging	Wiener et al. 1994, 1996 Raduchel et al. 1998

concanavalin A and pea lectin to yeast mannan (Page et al. 1996). The synthesis and properties of carbohydrate-containing dendrimers have been reviewed in many articles (Linhorst 1996; Roy 1996; Jayaraman et al. 1997).

Peptide-based dendrimers

The conventional approach to preparing anti-peptide antibodies is to conjugate a peptide to a known protein or synthetic polymer, in order to mimic the macromolecular structure of the native protein. However, this method generates macromolecular carriers that are ambiguous in structure and composition. To improve on this approach, Tam (1988) has developed multiple antigen peptide (MAP) systems as efficient and chemically defined systems to produce peptide immunogens in the absence of protein carriers. The MAP system is a polymer with a high density of surface peptide antigens and a Mw exceeding 10,000 Da. Using MAP as the basis, Nardelli et al. (1992) have attached peptide antigens derived from the envelope protein of the HIV-1 virus to the dendritic arms of the polymer and they found that these systems induced a strong antibody response that recognized the native proteins. As these peptide-based dendrimers showed great potential as vaccines, many synthetic approaches have been explored for example, Rao & Tam (1994) developed a method for synthesising peptide dendrimers that yields a large artificial protein (Mw 24,205 Da) and Shao & Tam (1995) investigated the use of unprotected peptides as buiding blocks for the synthesis of peptide dendrimers in aqueous media.

Targeting anticancer agent

Recently, PAMAM gen 3.5 has been used to prepare an anticancer conjugate with cisplatin (Malik 1999). After i.v. injection the PAMAM-cisplatin conjugate is able to selectively increase the platinum content of palpable B16F10 s.c. tumours approximately 50 fold compared to that seen of cisplatin at its maximum tolerated dose. This is due to passive localisation of the dendrimer-cisplatin in tumour tissue by the enhanced permeability and retention (EPR) effect (Malik 1999). Moreover, the toxicity of the conjugate was also less (3-15 fold) toxic than cisplatin.

Gene delivery

Recent advances in detecting inherited or acquired genetic disorders have provided the possibility of transferring recombinant genes into cells to correct missing or defective gene products. A variety of methods have been developed to accomplish gene transfer into eukaryotic cells. These techniques involve the direct physical introduction of genetic material into cells, the disruption of cell membranes to allow transfer of DNA, the use of genetically modified viruses to deliver genetic material, and the formation of DNA complexes with either inorganic salts, polycations, or lipids to transfer the DNA across

cell membranes. There is great utility for these techniques, but there are limitations in target cell type and in the ability to transfer different types of genetic material.

As cationic PAMAM dendrimers contain amino groups on their surface which are positively charged at physiologic pH, they can form complexes with biological polyanions, including nucleic acids. DNA-dendrimer complexes have been used to transfect cells in a similar manner to DNA-polylysine complexes but with better efficiency. The capability of DNA-dendrimer complexes to transfection cells was found to be dependent on dendrimer size, shape and the number of primary amino groups on their surface (Haensler & Szoka 1993; Kukowska-Latallo et al. 1996). Dendrimers with large diameter (> 40 nm) showed greater of transfection efficiency (Haensler & Szoka 1993). Transfection capability was restricted to PAMAM dendrimers of higher Mw (from ~22,000 to 700,000 Da) as a result of the higher number of surface amino groups (from 100 to 3,000) and the spherical shape (Kukowska-Latallo et al. 1996). These characteristics may allow for simultaneous interaction with negatively charged phospholipids on cell membrane, as well as the DNA. Significantly, no cytotoxicity was found during transfection performed on the 18 cell lines evaluation. Recently, a fifth gen PAMAM dendrimer, which is commonly used as gene delivery vehicle, was found to strongly activate complement (Plank et al. 1996). However, it was suggested that by appropriate formulation of DNA complexes, complement acivation could probably be minimized.

Biodegradable dendrimers

Any polymeric carrier designed for medical use should be preferably biodegradable. Most dendrimers used today such as PAMAM or DAB dendrimers are probably non degradable, but few quantitative have been undertaken probably because of the difficulty of chemical characterisation. Choice of appropriate size and Mw of each polymer is important to ensure the carrier will be harmless to the host. The first enzymatically degradable dendrimers have been derived from hydroxybutanoic acid and trimesic acid. They showed degradation in the presence of hydrolases, esterase, lipase and protease (Seebach et al. 1996). Others important properties of dendrimers include biocompatibility, biodistribution and immunogenicity. Most biological studies have been undertaken with PAMAM dendrimers and the results of these investigations are summarised below.

1.5.4. Dendrimers commonly used in drug delivery

Recent advances in chemical synthesis have generated a large number of novel dendrimers, but only few families of dendrimers are suitable for use in the pharmaceutical

field. PAMAM and poly(propyleneimine) dendrimers have been the most extensively studied.

PAMAM dendrimers

PAMAM dendrimers were first synthesised in the early 1980s by Tomalia and colleagues (Tomalia et al. 1985). The basic structure of there macromolecules is a repeating polyamidoamine unit usually terminating in amine surface (Figure 1.6), and they have been prepared from an ammonia core (NH_3) or ethylenediamine ($NH_2CH_2CH_2NH_2$) core. PAMAM dendrimers are synthesized by an alternating, sequential reaction involving addition of ethylenediamine and methylacrylate. This reaction produces a methyl ester intermediate via Michael's addition which is defined as a half gen. The addition of ethylenediamine produces a product with primary amine terminations and is termed a whole gen. Each complete reaction sequence give a new gen with a larger diameter (~ 10 Å per gen), twice the number of surface group (reaching 4,096 for a 10th gen) and with approximately a doubling in Mw (Tomalia et al. 1990).

PAMAM dendrimers have been produced commercially by Dendritech Inc. in large quantity and over a broad range of Mw. Defects that have been detected include branch defects, intramolecular looping, intermolecular bridging, intermolecular looping and intermolecular mixed bridging-looping (Tomalia & Durst 1993). Molecules without defects are monodisperse (index = 1.0) and molecules with defects are polydisperse (indices > 1.0). Size exclusion chromatography (SEC) is the technique of choice to determine the polydispersity index and the polydispersity of 1.01-1.08 has been reported for gen 0-9 (Tomalia et al. 1990). PAMAM dendrimers are generally sold commercially dissolved in methanol (~ 10 or 20% by weight), although they are soluble in water and other polar solvents. PAMAM dendrimers have been used as a support for antibodies, contrast agents, and radiopharmaceuticals and are underdevelopment for applications in a number of different areas of biology and medicine (Roberts et al. 1990; Barth et al. 1994; Singh et al. 1994; Wu et al. 1994).

The cytotoxicity of different gens of PAMAM dendrimers with an amine surface was studied using V79 Chinese hamster lung fibroblasts as a model. Cytotoxicity was found to be dose- and gen-dependent (Roberts et al. 1996). Furthur this observations was supported by the studies of Malik et al. (1999) who found that cationic PAMAM dendrimers were cytotoxic towards B16F10 cell lines and caused lysis of rat red blood cell (RBC). These studies suggest that larger cationic PAMAM dendrimers may not be a good choice for biological use whilst the smaller (gen 1) cationic PAMAM dendrimers appear to have little or no deleterious effects at least in these assays. In contrast, PAMAM

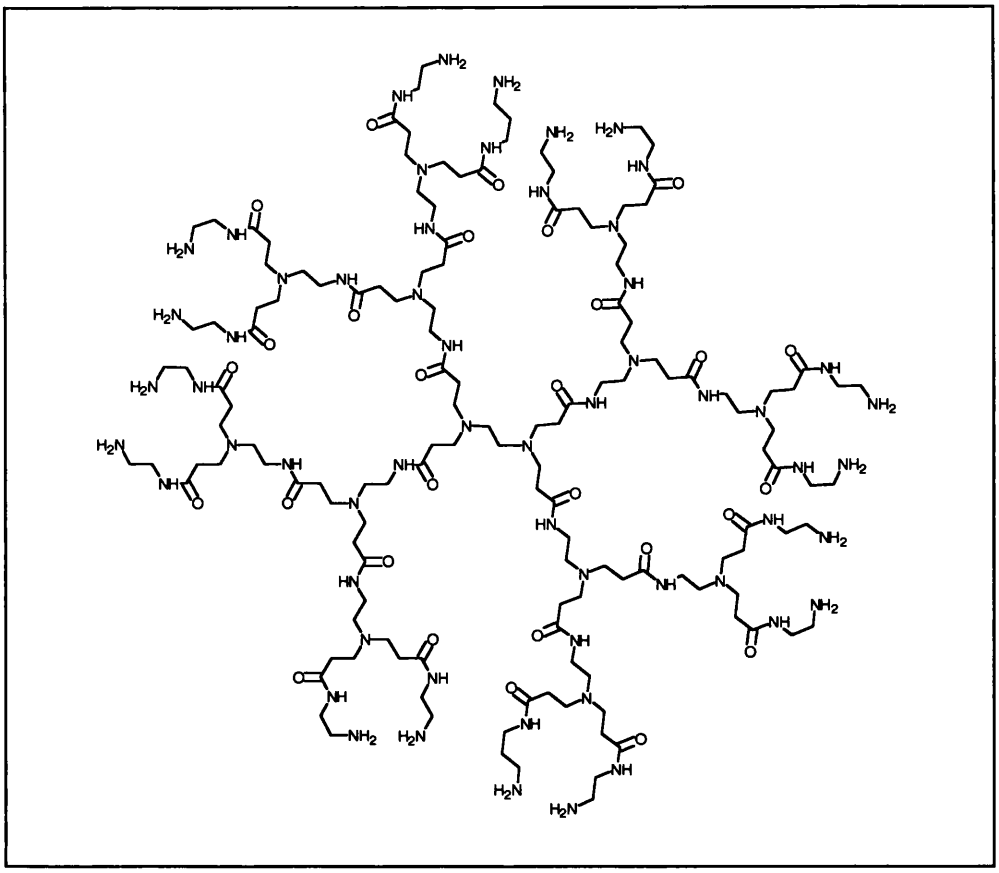


Figure 1.6. The structure of PAMAM dendrimers prepared using an ethylenediamine core

dendrimers bearing carboxylate surfaces were neither haemolytic nor cytotoxic toward cell lines in vitro (Malik et al. 1999).

In vivo studies have revealed that the biodistribution of PAMAM dendrimers is dependent on their size as well as their surface functionality (Roberts et al. 1996; Malik et al. 1999). After i.v. and i.p. administration cationic ^{125}I-labelled PAMAM dendrimers were readily cleared from the circulation. Only 0.1-1.0 % of the recovered dose was detected in blood at 1 h. Liver showed by far the highest levels of radioactivity at this time; 60-90 % of the recovered dose. Although the anionic ^{125}I-labelled PAMAM dendrimers displayed longer circulation times (15-40 % of the recovered dose in blood at 1 h) they also showed significant liver accumulation (25-70 % of the recovered dose) (Malik et al. 1999). Wilbur et al. (1998) also showed that ^{125}I-labelled iodobenzoate (PIB) - biotinylated-PAMAM dendrimers (gen 0-4) were cleared quickly with low blood levels (0.13-0.2 % dose/g) and higher kidney and liver levels at 4 h after i.v. administration. In this case the highest concentration of radioactivity was found in kidney (8-48 % dose/g). Surprisingly Roberts et al. (1996) reported that [^{14}C]gen 7 PAMAM dendrimer (Mw 87,000 Da) had an extremely high urinary excretion whereas the smaller dendrimer (gen 3, Mw 5,000 Da) accumulated in liver, kidney and spleen.

The suitability of dendrimers for parenteral administration in the clinical setting will ultimately be determined by their toxicity *in vivo* and also the toxicological profile of the drug payload that the dendrimer is designed to carry. Few *in vivo* toxicological studies involving dendrimers have been reported. Certainly PAMAM dendrimers bearing a carboxylate surface are less toxic than cationic derivatives. Three daily doses of PAMAM gen 3.5 i.p. at a dose of 95 mg/kg caused no adverse weight change in C57 mice bearing B16F10 tumours (Malik et al. 1999). In studies with cationic PAMAM dendrimers, Roberts et al (1996) administered gen 3, 5 and 7 to mice at maximum dose of 2.6, 10 and 45 mg/kg respectively. The dendrimers were given either as single dose or repeatedly once a week for 10 weeks. Although no behavioural changes or weight loss was reported administration of gen 7 did seem to have the potential to induce problem and 1/5 animals died. In the multiple dose study a degree of liver vacuolarisation was also observed during histopathology. These cationic PAMAM dendrimers were not immunogenic as measured by an immunoprecipitation and the Ouchterlony double diffusion assay (Roberts et al. 1996).

Poly(propyleneimine) dendrimers

Poly(propyleneimine) dendrimers are synthesised from a diaminobutane core, with addition of repeating polypropyleneimine units (de Brabander-van den Berg & Meijer 1993). They are also known as DAB dendrimers. The terminate functional groups

can be obtained in the form of amine, carboxylate or cyanoethylated forms (Figure 1.7). By the fifth gen, they have 64 surface groups, a molecular weight of approximately 7,000 Da and a diameter approximately 5 nm. These dendrimers are also readily miscible with water. The potential of DAB dendrimers as hosts for other molecules was demonstrated by Jansen and colleagues (1994) and this is discussed in Chapter 6.

There are only few studies relating to the biological properties of DAB dendrimers. The cytotoxicity with B16F10 melanoma cell lines demonstrated that the amine DAB were toxic even at low concentration as 100 μg/ml, whereas carboxylated DAB were generally nontoxic except at high concentration of 5 mg/ml (Malik et al. 1999).

1.6. Aims of this thesis

The aim of this thesis was to evaluate the potential use of dendrimers as an oral drug delivery system. First it was important to assess the biocompatibility of novel materials before conducting experiments *in vivo*. Different types of biocompatibility tests e.g. *in vitro* cytotoxicity, toxicity to rat intestinal tissue and red blood cell lysis were undertaken to evaluate the biocompatibility of two families of dendrimers diaminoethane (DAE) and PAMAM dendrimers, and also a family of branched polymer poly(vinylamine)(vinylformamide) (PVF) copolymers (Chapter 3).

After the evaluation of polymer biocompatibility, non-toxic PAMAM dendrimers were selected for further studies. Several potential uses of PAMAM dendrimers for oral delivery were studied systematically. First the potential of dendrimers to be orally absorbed was determined, PAMAM dendrimers of different surface and generation were radioiodinated and used to investigate their ability to traverse rat intestinal tissue *in vitro*. The effect of gen, surface charge, incubation time, and dendrimer concentration on the uptake into the tissue and across the tissue into serosal fluid were determined (Chapter 4). It was considered important to confirm the observations *in vitro* by study the *in vivo* GI transit time and body distribution after oral administration of ^{125}I-labelled PAMAM dendrimers to rats (Chapter 5).

Finally, the studies were designed to investigate whether dendrimers might be able to act as drug carriers for non-steroidal anti-inflammatory drugs (NSAIDS) and thus increase their water solubility and control release of drugs. Piroxicam and indomethacin were selected as model drugs and their complexation with dendrimers in aqueous solution was studied (Chapter 6). Also using ibuprofen as a model drug, the dendrimer-ibuprofen conjugates were synthesised and characterised (Chapter 7). The work presented in this thesis will provide the useful information for further development of these new

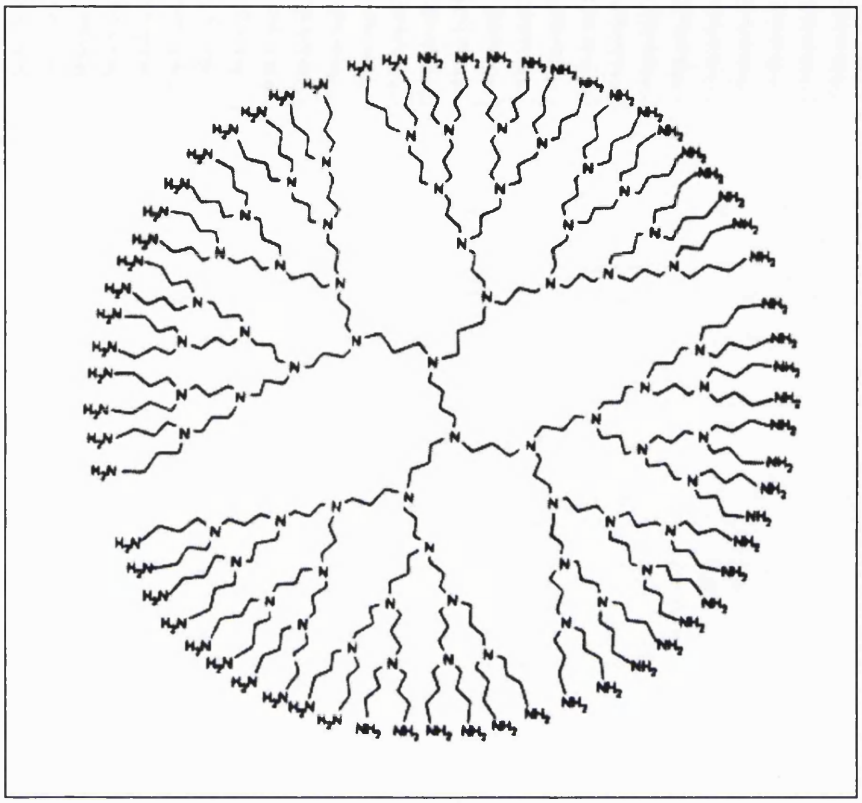


Figure 1.7. Molecular structure of DAB dendrimers (Jansen et al. 1994)

macromolecules as oral drug delivery systems and it is summarised in the last chapter of the thesis.

Chapter Two

Materials and Methods

2.1. Materials and equipment

Dendrimers and other polymers

Poly(vinylamine)(vinylformamide) (PVF) copolymers and diaminoethane (DAE) dendrimers were supplied by BASF (Germany). PAMAM dendrimers were purchased from Aldrich Chemical Company Ltd. (U.K.). Their structures and characteristics are shown in Figure 2.1-2.3 and Table 2.1-2.3, respectively.

Other materials and reagents

The following materials and reagents were obtained from Sigma Chemical Company Ltd. (U.K.): piroxicam, indomethacin, ibuprofen, dextran of Mw 74,000 Da, poly-L-Lysine of Mw 56,000 Da, polyethyleneimine (PEI) of Mw 70,000 Da, Triton-X-100, phosphate buffer saline (PBS), 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), optical grade dimethyl sulfoxide (DMSO), glutaraldehyde, osmium tetroxide, hexamethyldisilazane (HMDS), ethylenediamine, Medium 199 with Earle's salts, L-glutamine and sodium bicarbonate, o-dianisidine HCl, peroxidase, glucose oxidase, copper sulphate pentahydrate, sodium potassium tartrate, monobasic potassium phosphate and bovine serum albumin. Sodium dodecyl sulphate was from Bio-Rad Laboratories. Folin-Ciocalteau's reagent was from Fluka. Glucose, hydrochloric acid and sodium hydroxide were from BDH. RPMI 1640 and E199 medium were purchased from Gibco (U.K.). 1-Ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysulfosuccinamide (Sulfo-NHS) were from Pierce Warriner (U.K.). ^{125}I-Labelled Bolton and Hunter reagent was from Amersham International (U.K.). RS-ibuprofen [carboxyl-^{14}C] was from ICN Pharmaceutical (U.S.A.). All general reagents were from Sigma or BDH and were of analytical grade.

Cells and animals

B16F10 murine melanoma cells were obtained from Prof. Ian Hart, St Thomas' Hospital, London, U.K., L132 Human embryonic lung cells were from ECCAC (Wiltshire, U.K.). Wistar rats were from Banton & Kingman, U.K.

Equipment

UV/vis spectrophotometer (UV-1601, Shimadzu, Japan), Varifuge 3.0 RS centrifuge (Heraeus instrument, U.K.), Shandon paper electrophoresis tank (SLS, Nottingham, U.K.), Freeze dryer (FIS systems Flexi, U.K.), water bath OLS 200 (Grant U.K.), Titerteck plate reader (EFLAB, Finland), Rio-γ-counter 1274 (LKB-Wallack, London, U.K.), Liquid scintillation counter (Wallac 1409, Wallac, U.K.), Sephadex G-25 column (PD10) (Pharmacia Biotech, Hertfordshire, U.K.). Dialysis tubing Mw cut-off 2,000 Da (Spectrapor, U.K.). 717 Plus autosampler was from Waters (U.K.). C18

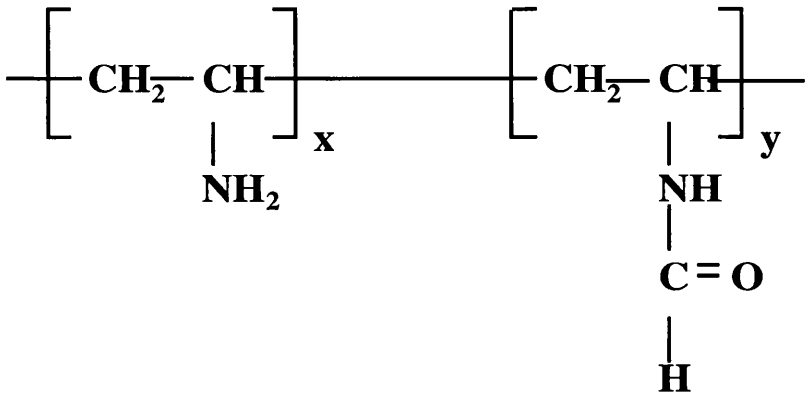


Figure 2.1. Structure of poly(vinylamine)vinylformamide copolymer

Table 2.1. Characteristics of poly(vinylamine)(vinylformamide) copolymer

PVF	Mw (Da)	Degree of hydrolysis*	pH**
1.1	30,000	28	7.75
1.2	30,000	49	7.70
1.3	30,000	92	7.19
2.1	300,000	32	8.51
2.2	300,000	51	8.08
2.3	300,000	92	7.13
3.1	1,000,000	30	8.25
3.2	1,000,000	50	7.77
3.3	1,000,000	90	7.04

* The values represents the conversion of polyvinylformamide to polyvinylamine

** pH of PVF 1 mg/ml in phosphate buffer saline (PBS)

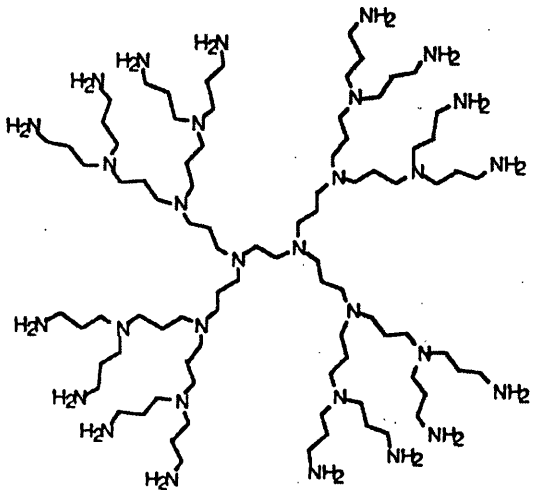


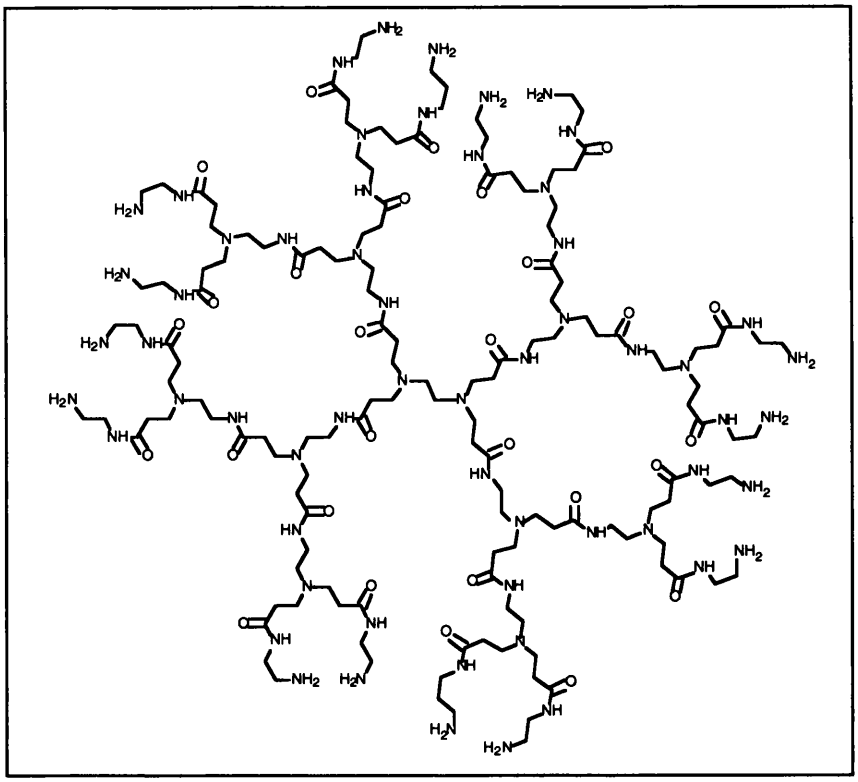
Figure 2.2. Structure of DAE dendrimer

Table 2.2. Characteristics of DAE dendrimers

Dendrimer	Mw (Da)	No. of terminal groups
DAE 4	289	4
DAE 8	745	8
DAE 16	1659	16
DAE 32	3486	32
DAE 4/1*	233	4
DAE 4/2**	303	4

** contain 2C in the branch chain.*
*** contain propylenediamine (3C) in the core.*

(a)



(b)

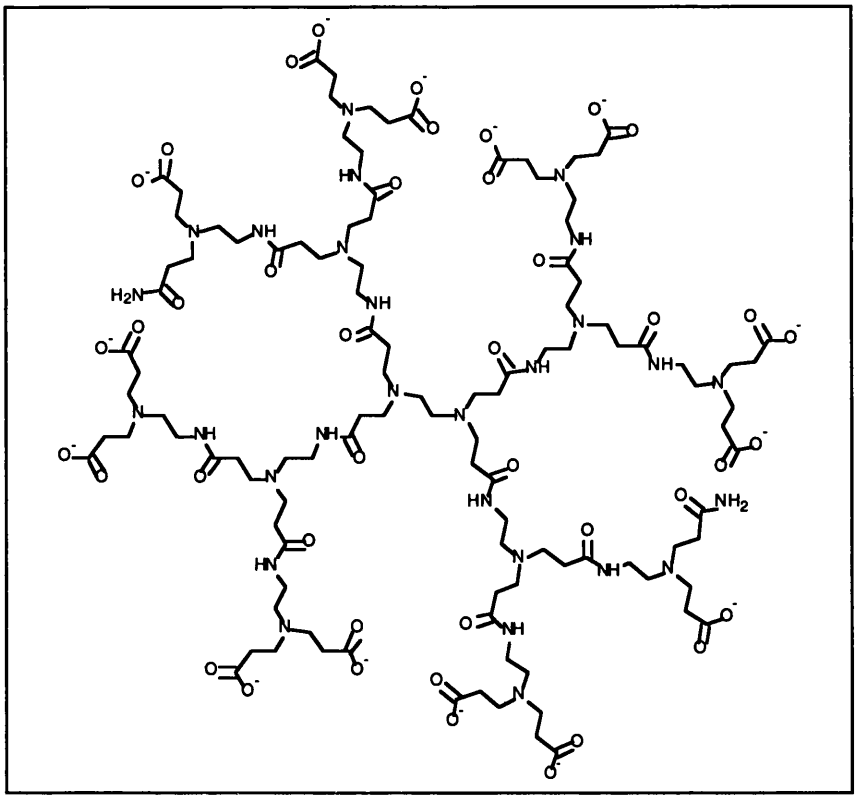


Figure 2.3. Structure of PAMAM dendrimers
(a) Amine terminated (b) Carboxylate terminated

Table 2.3. Characteristics of PAMAM dendrimers

Generation	Terminal groups		Mw	Diameter
	Type	Number	(Da)	(nm)
3	NH_2	32	6,909	3.6
4	NH_2	64	14,215	4.5
2.5	COO^-Na^+	32	6,011	3.6
3.5	COO^-Na^+	64	12,419	4.5
5.5	COO^-Na^+	256	50,865	6.7

Bondapak column (150 x 3.9 mm), pump (LKB Bromma 2150), UV detector (Spectrflow 783, Kratos analytical). All data from liquid chromatography experiments were analysed by Powerchrom programme (version 2.0.7).

2.2. Methods

Biocompatibility Evaluation

To investigate the biocompatibility of dendrimers and other polymers experiments were undertaken to determine their ability to induce haemolysis, cause cytotoxicity to cells in culture and damage everted rat intestinal tissue. The methods are summarised as follows:

2.2.1. RBC lysis assay

Fresh blood was obtained from male Wistar rats (~250 g body weight) by cardiac puncture following CO_2 asphyxiation and was collected in a heparin/lithium blood tube. RBC were isolated by centrifugation at 1,000 x g for 10 min at 4°C, then the supernatant and the top few mm of cell layer was aspirated off (Sgouras 1990). The RBC were resuspended in chilled PBS (4°C) and recentrifuged at the same speed and temperature for 10 min, then the supernatant was again removed. Following the repeating washing process (3 times) the final RBC pellet was resuspended in PBS to give a 2% w/v suspension.

Then different concentrations of dendrimer and polymer (0-5 mg/ml) were prepared and added to a microtitre plate, 100 µl each well (8 replicates of each dilution). Reference standards; dextran and PEI were prepared and added in the same manner. Triton X-100 (1% v/v) was used to provide 100% RBC lysis and this was added to the rest of the wells (Duncan et al. 1994). At the start of the experiment RBC (2% w/v) suspension 100 µl was added and the plate incubated at 37°C for 1 h or 5 h. After incubation, the plates were centrifuged at 1,500 x g for 10 min at room temperature and the supernatant was decanted into another microtitre plate in order to read the absorbance at 550 nm using a Titerteck plate reader. The data obtained were expressed as the percentage haemoglobin released relative to the total release caused by Triton X-100.

2.2.2. In vitro cytotoxicity

Maintainance of cell lines: Cells were maintained in an atmosphere of 5% v/v CO_2 at 37°C in a humidified CO_2 culture incubator. All cell culture techniques were carried out in a class II Laminar flow cabinet. All material added to cell cultures was sterile,

osmotically balanced and warmed to 37°C in a water bath for approximately 30 min prior to use. Cells (B16F10 and L132) were maintained in the tissue culture media listed in Table 2.4. The cells were maintained in 75 cm^2 tissue culture treated, cantered neck flasks with vented (0.2μm) tops and were passaged once every 7 days. The medium was replaced every 3-4 days to avoid depletion of essential nutrients. This maintained the cells in the exponential phase of growth.

Regeneration of frozen cultures: Upon arrival, cells were stored frozen in a vial. To recover cells the cryogenic vials were placed at 37°C inside a 30 ml sterile universal bottle until they were visually seen to thaw (approximately 5 min). Then the cell suspension was subject to centrifugation at 1,500 x g for 10 min at 22°C. The supernatant was then removed and the cells re-suspended in the appropriate cell culture media (10 ml) (Table 2.4) and used to seed a 75 cm^2 flask.

Passage of cells: To passage cell cultures, first the culture media was removed by aspiration and the cells washed twice with PBS (10 ml). The cells were removed from the flask by the addition of trypsin (1 ml) and the flask was placed in the incubator until most of the cells had detached. Following incubation (approximately 3-5 min) and gentle agitation, the monolayer was examined microscopically using an inverted microscope. Once the majority of the cells were seen in suspension, media (9 ml) containing 10% FCS was added to dilute the trypsin. Aliquots of the suspension were used to seed further flasks at a density dictated by the generation time and hence split ratio of the cell line (Table 2.4).

Cell number determination: To measure cell number in order to establish the growth curve and to allow seeding of an appropriate density of cell to each well for cytotoxicity assays it was necessary to determine cell number. Cells in suspension (prepared as described above) were counted using an improved Neurbauer haemacytometer slide. After placing a coverslip on the haemacytometer approximately 5 μl of cell suspension was placed into the counting chamber. Cell number were assessed by counting the average number of cells in a known volume (0.1 x 0.1 x 0.1 mm). This value was multiplied by any dilution factor used and then multiplied by 1 x 10^4 to give the number of cells present / ml.

MTT assay to assess cell viability: The MTT assay was used to assess cell viability to measure either the cell growth characteristics or the cytotoxicity of added compounds. The MTT assay works on the principle that the mitochondrial dehydrogenase

Table 2.4. Cell lines and culture conditions

Cell line	Name	Split ratio	Supplier	Culture media
B16F10	Murine malignant melanoma	1 : 20	Prof. Ian Hart St Thomas's hospital London, UK	FCS (10 % v/v) , RPMI 1640 25 mM HEPES 5 mM L-glutamine
L132	Human embryonic lung	1 : 10	ECCAC	E199, 5 mM L-glutamine FCS (5% v/v)

enzyme found in viable cells is able to metabolise the water soluble tetrazolium dye MTT into an insoluble purple formazan salt (Mossman 1983; Sgouras 1990; Liu et al 1997). The reaction is shown in Figure 2.4.

To determine the cell growth curve, cells were seeded into a sterile, flat bottomed 96 well microtitre plate at a density of 1 x 10^3 cells/well (100 µl/well). The plate was then placed in the 5% CO_2 incubator at 37 °C. MTT was made up to a 5 mg/ml solution in PBS and filter sterilised through a 0.2 µl disposable filter (Acrodisc). At 5 h prior to the end of the incubation period 20 µl of MTT stock was added into each of 8 wells in a row of the plate and the cells were left for 5 h. At the end of the incubation period i.e. 24, 48, 72, 96, 120, and 168 h, the MTT media was removed and the insoluble formazan crystals were dissolved in 100 µl of optical grade DMSO. The plates were read in a Titerteck plate reader at 550 nm after blanking with DMSO. The cell growth curve was constructed by plotting the absorbance against time. Growth curves for B16F10 and L132 cells are shown in Figures 2.5a and 2.5b respectively, and they were used to determine the cell doubling time by calculating the time taken for the absorbance to double on the linear part of the curve.

To study cytotoxicity of the dendrimers and other polymers, poly-L-lysine was used as a positive control and the dextran was used as a negative control. These polymers have been used as a reference before (Sgouras 1990; Duncan et al. 1996). B16F10 or L132 cells in their logarithmic phase of growth were seeded at a density of 1x10^5 cells per ml (1x10^4 cells per well) in a 96 well flat bottomed microtitre plate. The cells were left for 24 h in the 5% CO_2 incubator at 37°C to readhere. All polymers were dissolved in culture media (Table 2.4) and then sterilised through a 0.2 mm sterile filter, the first few microlitres of the solution being discarded in the case of adherence of the polymer to the filter membrane. Then dendrimers and the reference polymers were added in increasing concentrations (1-5 mg/ml) to the cells in the microtitre plate. The cells were left in the incubator for 67 h then MTT was added as previously described for growth curve. The same procedure was used for MTT. The results expressed as a percentage of viability compared to that observed in cell grown without addition of polymer. (Sgouras & Duncan 1990).

2.2.3. Scanning electron microscopy (SEM)

To visualise cells by SEM, RBC were incubated with DAE dendrimers and reference polymer PEI at 10 µg/ml and 1 mg/ml (1 h) or B16F10 cells grown at a cell density of 1x10^5 per ml on coverslips in a 6 well tissue culture plate in the presence of dendrimer (same concentrations) for 1h. In both cases cells were then harvested, washed

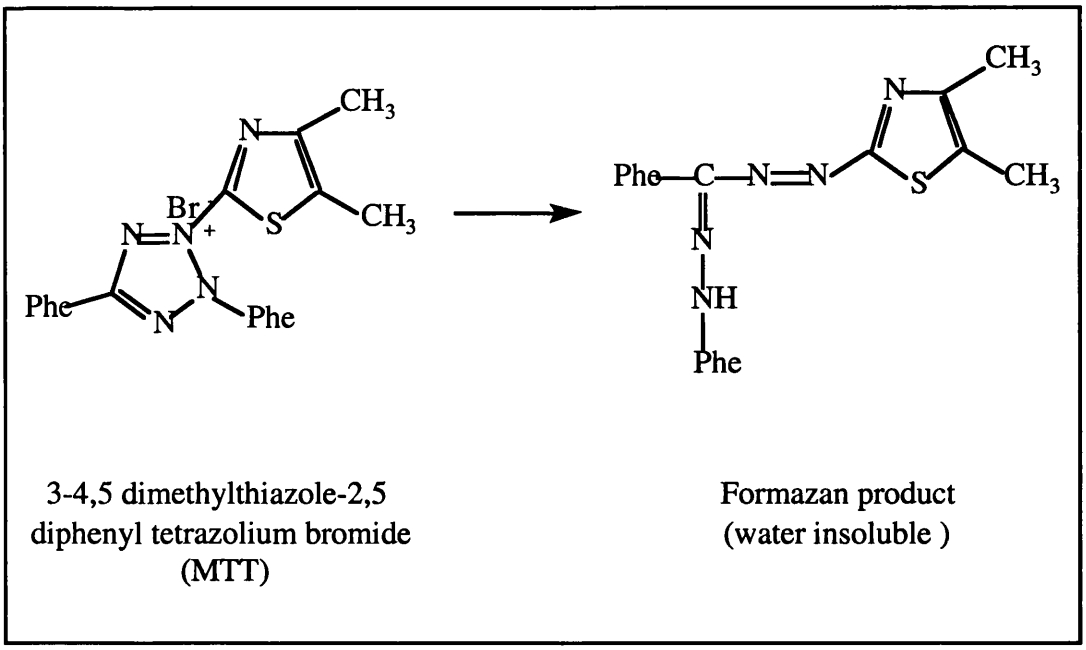
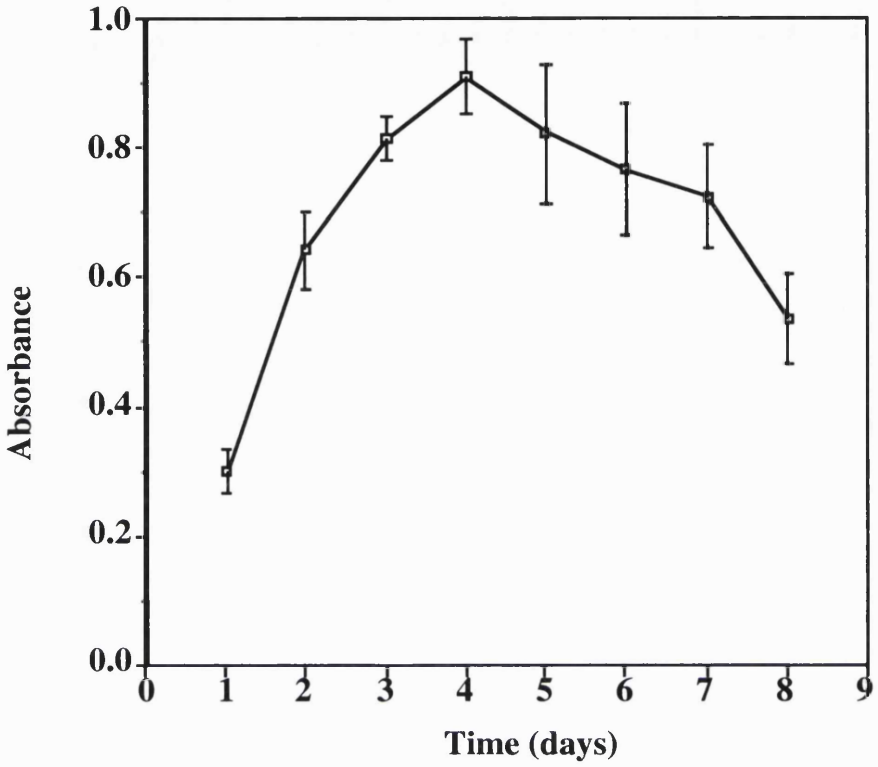


Figure 2.4. The MTT reaction

(a)



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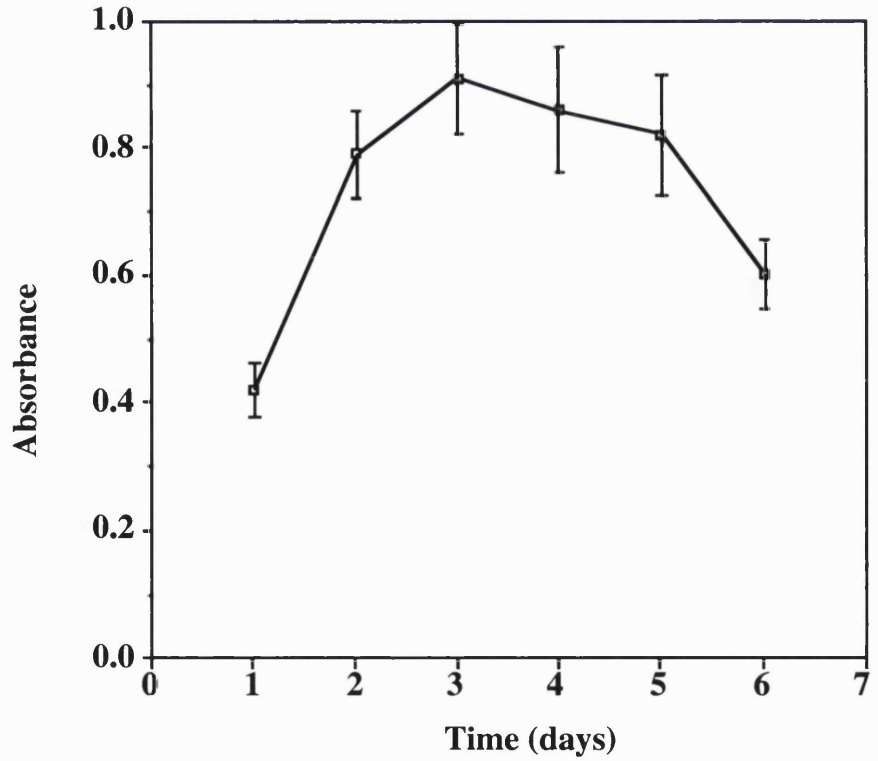


Figure 2.5. Growth curve for (a) B16F10 murine melanoma cell and (b) L132 human embryonic lung cell

twice with PBS, and then placed in 1 ml of SEM grade glutaraldehyde (2.5% v/v) for 24 h. After washing with PBS cells were placed in 1% w/v osmium tetroxide and left for 1 h before step-wise dehydration in increasing concentration of ethanol solutions and a final wash with 100% ethanol. Cells were re-suspended in HMDS and placed onto glass coverslips. After the evaporation of HMDS, gold deposition was performed using a K550 coater set for 5 min at 50 μA.

2.2.4. Toxicity towards the intestinal tissue

To investigate the toxicity of dendrimer towards rat intestinal tissue, everted sacs were prepared according to the method of Woodley and colleagues (Naisbett & Woodley 1994). Tissue culture medium 199 contains glucose at concentration of 1 mg/ml and thus active transport of glucose with time (medium to serosal) can be used as a measure of tissue viability. Adult male Wistar rats (250-350 g, 10-12 week old) were fasted overnight and killed by cervical dislocation. The small intestine was rapidly removed and placed in medium 199 oxygenated with a 95% O_2/ 5% CO_2 mixture and kept at 37°C. After washing through with saline (0.9% w/v NaCl) the tissue was everted over a glass rod (2.5 mm in diameter) and was filled with oxygenated medium 199 (37°C). To prepare sacs, approximately 2.5 cm of the small intestine without visibly containing Peyer's patches was tied off using surgical silk. Each sac was immediately placed in a stoppered Erhlenmeyer flask containing 10 ml of different concentrations of dendrimer (0-100 μg/ml) in oxygenated medium 199 and placed in a shaking water bath at 37°C. Following the 90 min incubation, the sac was cut open, the serosal fluid (liquid inside the sac) (20 μl) and a sample of culture medium (20 μl) were collected and the glucose concentration in both samples determined using a modification of the method by Dahlqvist (Dahlquist 1968). Samples were incubated at 37°C with 1 ml containing glucose oxidase reagent (Triton-X100 (0.2% w/v in ethanol), o-dianisidine-HCl (10 μg/ml), peroxidase (1 μg/ml), glucose oxidase (200 μg/ml) in 0.5 M Tris/HCl, pH 7.2). The reaction was terminated by the addition of 2 ml of 5 M HCl, and the absorbance was measured at 525 nm. The glucose concentration was then estimated using a glucose standard curve prepared according to the same protocol.

Radioiodination of PAMAM dendrimers and investigation of their pharmacokinetics *in vitro* and *in vivo*

In order to study the pharmacokinetic of PAMAM dendrimers both *in vitro* and *in vivo*, the dendrimers have been labeled using ^{125}I-labelled Bolton-Hunter reagent. Dendrimers with amine surface were directly coupled to the reagent while the dendrimers with carboxylate surface were first modified by introduction of an amine group into the

molecule before the coupling reaction. The ^{125}I-Labelled PAMAM dendrimers were obtained from Dr. Malik and the detail of radiolabelling method has been described elsewhere (Malik 1999).

2.2.5. Introduction of an amine into PAMAM dendrimers

Anionic dendrimers (gen 2.5, gen 3.5 and gen 5.5) were supplied in methanol (10%w/v) as a sodium salt. Samples (10 mg) were first dried under a stream of nitrogen to give a solid residue and then re-dissolve in double distilled water (DDW) to give a final concentration of 10 mg/ml. The pH was monitored and adjusted to 6.5 with dilute HCl. EDC (a molar ratio sufficient to modify one carboxylate residue per dendrimer) was added and the reaction left stirring for 30 min at room temperature. Ethylenediamine (a molar equivalent to EDC) was then added slowly to prevent crosslinking. The reaction was left for 4 h and unreacted EDC removed by dialysis. The ninhydrin assay was used to verify the number of amino groups on the surface of the anionic-modified dendrimers (Plummer 1978).

2.2.6. ^{125}I-Radiolabelling of PAMAM dendrimers using the Bolton and Hunter reagent

The cationic PAMAM dendrimers gen 3 and gen 4 (10 mg), and the anionic dendrimers gen 2.5, gen 3.5 and gen 5.5 modified with ethylenediamine (20 mg) were dissolved in borate buffer 0.5 ml (pH 8.5, 0.1 M). ^{125}I-Labelled Bolton-Hunter reagent (0.5 mCi; 100 µl in benzene) was carefully dried under a stream of nitrogen. The dendrimer solution was then added and allowed to react for 15 min on ice, mixing periodically. A sample (5µl) of the reaction mixture was removed for analysis and the remaining solution was carefully purified by dialysis against NaCl (1% w/v). The ^{125}I-labelled dendrimer preparations were then stored at 4°C until use. The labelling efficiency and percentage of free [^{125}I]iodine in each sample was determined by paper electrophoresis. The structure of Bolton-Hunter reagent and the ^{125}I-labelled PAMAM dendrimer are shown in Figure 2.6.

2.2.7. Characterisation of radioiodinated dendrimers by paper electrophoresis

It was necessary to determine the labelling efficiency of each dendrimer and ensure that the preparation used to measure transport did not contain free [^{125}I]iodide or free ^{125}I-labelled Bolton-Hunter reagent. An electrophoresis tank was filled with barbitone buffer (50 mM sodium barbital and 10 mM barbital). Whatman No.1 chromatography paper was cut into 5 x 30 cm sized strip and the central portion divided into 40 x 0.5 cm strips by pencil lines. The paper was moistened with the barbitone buffer and placed

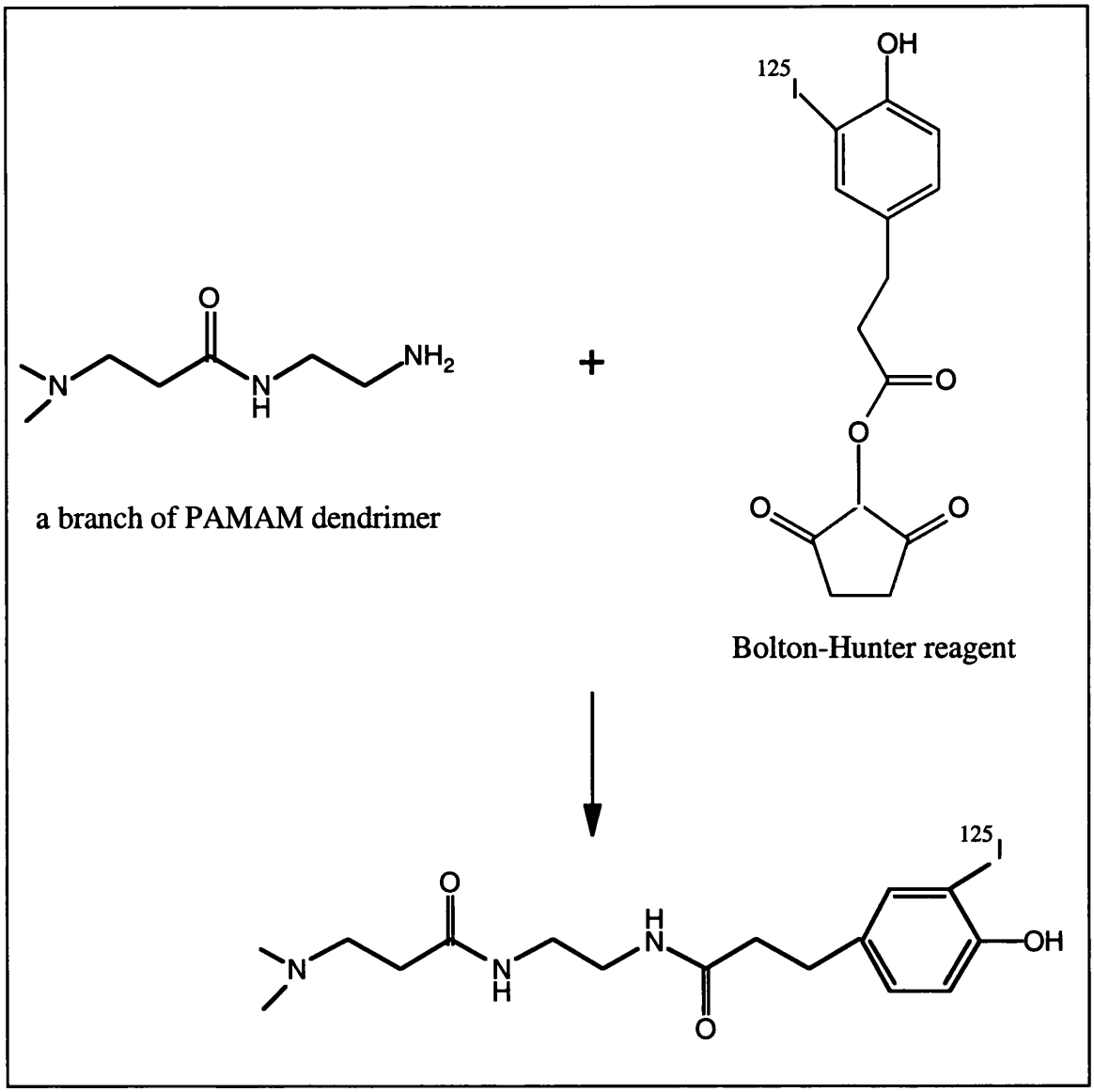


Figure 2.6. Radioiodination of PAMAM dendrimers used the Bolton-Hunter reagent. (The diagram shows only one branch arm of the dendrimer. The full structures are shown in Figure 2.3)

across the supporting bars. The reaction mixture, purified radioiodinated dendrimer (5 µl) and free sodium [^{125}I]iodide were applied to strips on the fifth strip marked onto the paper which was near the anode. A voltage of 400 V was passed through the paper for 30 min. Then, the paper was removed and cut into individual strips that were then submerged in 1 ml of water in counting tubes. The tubes were counted for radioactivity using a γ-counter. The results were plotted as counts per minute (cpm) against distance migrated. The specific activity was calculated for each radiolabelled dendrimer (expressed in term of µCi / mg) from the electrophoretic pattern obtained from the reaction mixture.

2.2.8. Uptake and serosal transport of ^{125}I-labelled PAMAM dendrimer by the rat small intestine in vitro

Everted sacs (prepared as above) were incubated with ^{125}I-labelled PAMAM dendrimers for periods up to 2 h and at concentrations 20 µg/ml (shown non-toxic). At each sample time (every 30 min), sacs were removed, washed 3 times with saline and blotted dry. Sacs were then weighted, cut opened and the serosal contents were carefully collected. Sacs were re-weighed after draining to accurately calculate the volume inside each sac. The individual sac tissues were digested in 5M NaOH (5 ml) by incubation at 37°C for 3 h. Samples (1 ml) of the serosal fluid, incubation medium and the tissue digest were assayed for radioactivity (Figure 2.7). The tissue digest was also analysed for protein content using the Lowry method as modified by Peterson (1983). The uptake of ^{125}I-labelled dendrimer by tissue and the serosal transfer was expressed either in terms of ng dendrimer/mg sac protein or as the clearance rate termed the "Endocytic Index" (EI) The EI expresses uptake as the equivalent volume of culture medium (µl) whose contained substrate is captured or transported/mg tissue protein/h. The EI is useful as it can be used to allow comparison of the rate of tissue uptake or transport of different substrates (Williams et al. 1975).

To study the concentration dependence of dendrimer uptake, everted sacs were incubated with different concentration of ^{125}I-labelled dendrimer (0-100 µg/ml) for 1 h. and the determination process was repeated the same as above.

To determine the protein content of tissue digest, samples from tissue digest (25 µl) were made up to 1 ml with distilled water and dispensed into individual test tubes, along with 1 ml of the 25, 50, 100 and 200 mg/ml standard bovine serum albumin solutions and a blank containing 1.0 ml of distilled water. Then reagent A (1 ml) (a mixture of CTC reagent (1 part), SDS (5% w/v) (2 parts) and 0.8M NaOH (1 part) was added into each tube. The tubes were then mixed and allowed to stand at room temperature for 10 min. After that, reagent B (0.5 ml) (0.22M Folin-Ciocalteu phenol

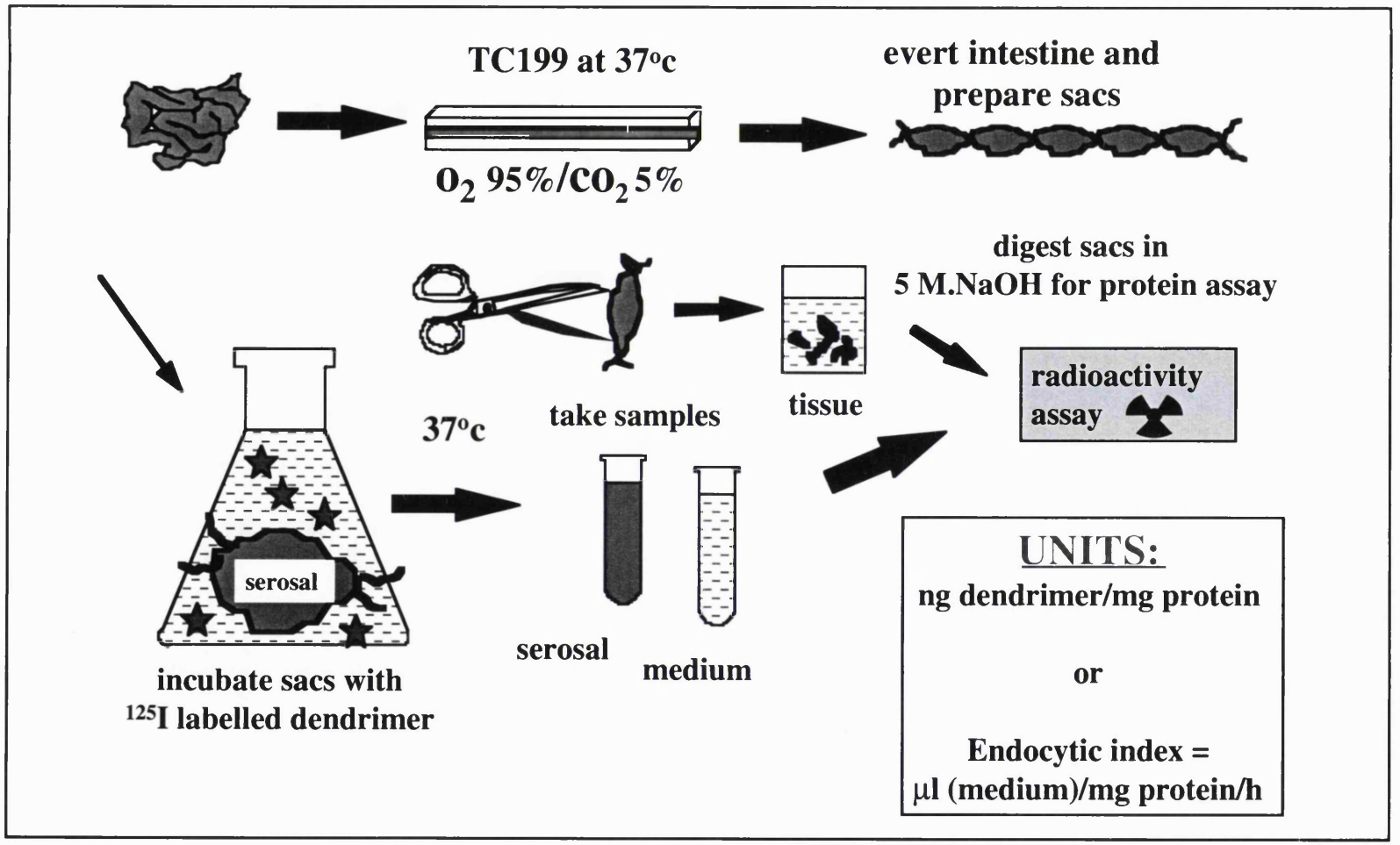


Figure 2.7. Method used for the preparation of the everted sacs and the determination of uptake of ^{125}I-labelled dendrimers

reagent) was added, the tubes were mixed and allowed to stand for another 30 min and then the absorbance were read at 750 nm. The total mg tissue protein of each sac was then calculated from the standard curve.

Along with the uptake experiment in each sac, sac viability can be detected by the ability of the sacs to transport glucose. Glucose concentration in the incubation medium and in the sac was measured as the same procedure above.

To analyse the nature of the radioactivity recovered in the serosal fluid after the uptake and transfer process, the serosal contents of 3-6 sacs incubated for 2 h were pooled and freeze dried. The resulting residue was resuspended in 1.0 ml of medium 199 and analysed by sephadex G-25 column chromatography. First the column storage solution was drained off and the column equilibrated with 25ml PBS. The sample was then loaded on to the column (1 ml) and eluted by PBS. The fractions (0.5ml) were collected into Luckham tubes and counted using a γ-counter. The samples of the culture medium (1.0 ml) after 2 h incubation were also characterised by sephadex G-25 column chromatography in the same manner. The composition of both the serosal and medium samples was compared with the original elution profile of the ^{125}I-labelled PAMAM dendrimer.

2.2.9. Uptake and serosal transport of free [^{125}I] iodide by the rat small intestine in vitro

The uptake and transfer of sodium [^{125}I]iodide at the amount of 650 cpm in medium (equivalent to 2-10% of total radioactivity of radiolabelled dendrimer in the medium) was studied over 120 min using the same procedure as above. Effect of concentration of free [^{125}I]iodide (0-1300 cpm) on the accumulation in tissue and serosal was also determined after 1 h incubation.

2.2.10. In vivo body distribution and GI transit of ^{125}I-labelled PAMAM dendrimers in rats

After measurement of the uptake and serosal transport of the radiolabelled PAMAM dendrimers *in vitro*, it was considered important to study further the GI transit and body distribution of these compounds after oral administration *in vivo*.

^{125}I-Labelled PAMAM dendrimers were administered (5 mg/kg) to the adult male Wistar rats (250-300 g) using a blunt tipped feeding needle inserted into the stomach. The animals, which had free access to water, were fasted for 12 h prior to dosing and food was returned 2 h later. The animals were kept in metabolic cages (with food and water) to

facilitate collection of urine and faeces over time. At time intervals of 1, 5 and 24 h the rats (n=3) were sacrified, blood was collected (2 x 500 µl) from the heart, the following organs: liver, spleen, kidney, heart, lung, thyroid gland, stomach, small intestine, caecum and colon were removed, washed with saline (NaCl 0.9% w/v) and blotted dry. Samples were weighted and homogenised in distilled water to a known volume. For stomach, small intestine and colon, the washings from the lumen were also collected. Faeces were suspended in water and homogenised. The organ homogenates, GIT washing, urine and faeces homogenates were counted for radioactivity in a γ-counter. The radioactivity recovered was expressed as a percentage of the total dose recovered. The total blood volume of the rat was calculated assuming 6.3 ml per 100 g body weight (Baker et al. 1979).

To examine the nature of the radioactivity recovered, samples of urine and faeces collected after 24 h were analysed by sephadex G-25 column chromatography as above (section 2.2.8). Urine samples (1 ml) were applied directly to the column using PBS. Faeces samples were digested in 5 M NaOH for 5 h, centrifuged at 3000 x g for 30 min, the supernatant was then applied to a sephadex G-25 column. The resulting fractions were assayed for radioactivity in a γ-counter.

To study the stability of ^{125}I-labelled dendrimers in GI fluid, the radiolabelled dendrimers (1 ml) were incubated in simulated gastric fluid pH 1.2 USP (9ml) for 1 h or in simulated intestinal fluid pH 7.5 USP (9ml) for 5 h. All experiments were performed in shaking water bath at 37°C. After a given incubation time, 1 ml of sample was taken and subjected to gel permeation chromatography using the sephadex G-25 column. The elution profile obtained was compared with the elution profile of the starting preparation of ^{125}I-labelled dendrimer and the percentage of degradation product was then calculated.

Solubilization and complexation of nonsteroidal anti-inflammatory drugs with dendrimers in aqueous solution

The intramolecular core of dendrimer provides the opportunity to entrap small molecules inside (Jansen et al. 1994) which subsequently may improve the following properties of drug:- water solubility, stability, membrane permeability, and bioavailability. In addition the controlled release of drug is possible. To investigate the possibility of complexation of drugs with PAMAM dendrimers, the following topics were studied:- the ability of PAMAM dendrimers to increase water solubility of drugs, acid-base properties of dendrimers and the interaction of drug with dendrimers in aqueous solution. Two nonsteroidal anti-inflammatory agents piroxicam and indomethacin, both

of which are very slightly soluble in water, were used as model drugs in these experiments.

2.2.11. Solubility study of piroxicam and indomethacin in presence of dendrimers

Before examining the ability of dendrimers to solubilise piroxicam and indomethacin, the calibration curves of both drugs in buffer solutions were prepared as follows. A 0.002% w/v stock solution of piroxicam or indomethacin was made up in a mixture of 1 volume of methanol and 11.5 volume of buffer (HCl buffer pH 2, phosphate buffer pH 6 and borate buffer pH 8). Serial dilutions of these solutions were then prepared and measured for the UV absorbance at the appropriate wavelength (356 nm for piroxicam and 316 nm for indomethacin) using buffer solution as a blank solution. The results was recorded and plotted as absorbance against drug concentration (Appendix II).

PAMAM dendrimers (gen 2.5 or gen 3) were prepared at different concentrations (0-5 mg/ml) in HCl buffer solution pH 2 (0.2 M), phosphate buffer solution pH 6 (0.2 M) or borate buffer solution pH 8 (0.2 M). Vials were prepared containing 5 ml of these solutions (in triplicate) and the pH of solution was then measured (dendrimers can change the buffer pH). An excess amount of piroxicam or indomethacin (25 mg) was added to each vial and each sample was sealed and placed into a shaking water bath at 25°C at a rate of 70 rotations per min. After 24 h the samples were removed and filtered using a Sartorious membrane filter, pore size 0.2 μm, in order to remove insoluble excess drug. The filtrates were then diluted and the absorbance measured using a UV spectrophotometer set at the appropriate wavelength (356 nm for piroxicam and 316 nm for indomethacin). The drug concentration was calculated using the appropriate calibration curves. To investigate whether dendrimers caused any change in the absorbance spectrum of drug, each sample was also scanned in the 200-500 nm range. Solutions of dendrimer alone were also scanned to see if there is any absorbance at the wavelength used.

To investigate the influence of the pH on the ability of dendrimers to solubilise drug, the pH-dependence of drug solubility in the presence of dendrimers was investigated using the same procedure as above and phosphate buffers of pH 6.0-9.0 and borate buffers pH 8.0-10.0.

2.2.12. Preliminary study on the solubility of napthalene in presence of dendrimers

To investigate whether the intramolecular core of PAMAM dendrimers can interact with a guest molecule by hydrophobic interaction, the solubility of napthalene was assessed in the presence of dendrimers. PAMAM dendrimers (gen 2.5 or gen 3) were prepared at concentrations 0 and 5 mg/ml in DDW (5 ml). An excess amount of napthalene (20 mg) was added to each vial and each sample was sealed and placed into a sonication bath at 25°C. After 3 h the samples were removed and filtered using a Sartorious membrane filter, pore size 0.2 μm, in order to remove insoluble excess napthalene. The filtrates were then diluted and the absorbance measured using a UV spectrophotometer at 284 nm using dendrimer solution as a blank (dendrimer has absorbance in this wavelength). The drug concentration was calculated using their calibration curve.

2.2.13. Determination of acid-base properties of PAMAM dendrimers

To have better understanding of acid-base properties of PAMAM dendrimers, the titration curves for dendrimers with a carboxylate surface (gen 2.5 and gen 3.5) and dendrimer with an amine surface (gen 3 and gen 4) were produced.

A solution of each dendrimer (200 mg) in potassium chloride solution (0.5 M, 10 ml) was prepared. All samples were titrated with HCl (1.0658 M) in duplicate. The titration curves were constructed by plotting the volume of acid added against pH. From the titration data, the number of charged groups per mole of dendrimer was calculated using equations described in Chapter 6. This was plotted against pH. The pKa and end points of protonation for each gen were then estimated from the curves.

2.2.14. Release of piroxicam and indomethacin from PAMAM dendrimer complexes

To study the release of piroxicam and indomethacin from dendrimer complexes, first the complexes were prepared as follows. PAMAM dendrimer (gen 2.5 or gen 3) were dissolved in phosphate buffer pH 6 or borate buffer pH 8 at a concentration 3 mg/ml. Piroxicam or indomethacin was added and dissolved in dendrimer solution to give a mole ratio of dendrimer to drug of 1:1 at pH 6 and 1:10 at pH 8. Each sample (1 ml) was placed in a dialysis tube (Mw cut-off 2,000 Da) which was then placed into an Ehrlenmeyer flask containing the same buffer solution (50ml). This was left in a shaking water bath at 37°C with 70 rotations / min. Samples (1 ml) of dialysis fluid were taken at each time point up to 2 h the sample was replaced by the same amount of fresh buffer solution. As a control, the experiment was also performed using drug in buffer solutions

without dendrimer. The percentage of drug release across the dialysis membrane was then calculated and plotted against time.

Synthesis and characterisation of PAMAM dendrimer-ibuprofen conjugates: A preliminary study

2.2.15. Synthesis and characterisation of conjugates

Ibuprofen was conjugated to PAMAM dendrimer gen 3 via an amide linkage using EDC and sulfo-NHS as coupling agents. PAMAM gen 3 (100 mg, 0.015 mmole) was first dried under a stream of nitrogen to a solid residue and then re-dissolve in double distilled water (2 ml). Ibuprofen, to give the ratio of 10, 15 and 20 moles of drug to 1 mole of dendrimer, was dissolved in DMF (2 ml) which contained EDC and sulfo-NHS (0.5 mole : 1 mole drug). To follow the reaction and characterise the conjugate the small amount of [^{14}C]ibuprofen was also added to the DMF mixture (ratio of ibuprofen : radiolabelled ibuprofen is 1 : 7,500) and this was left stirring at room temperature for 30 min. After activation of ibuprofen by EDC, the DMF mixture was then added dropwise to the dendrimer solution and the reaction was left stirring for 4 h.

To remove free drug and other low Mw compounds, the conjugate was purified by passing through the sephadex G-25 column using PBS as an eluent. All the collected fractions were measured for the radioactivity using a liquid scintillation counter. The elution profile of conjugate was compared with the original profile of free [^{14}C]ibuprofen and dendrimer by its UV absorption. The fractions which contained conjugate were then pooled and freeze-dried. Following the purification and characterisation by monitoring the radioactive ibuprofen. The mole ratio of drug to dendrimer can be approximately calculated from the area under curve. The structure of the conjugate was confirmed by the ^{1}H NMR profile.

2.2.16. Determination of the free drug content in the conjugates

Prior to determination of free ibuprofen in the conjugates, the standard curve of ibuprofen was prepared (0.01 - 0.05 mg/ml) in DDW (1 ml), extracted and quantified by HPLC as described in Chapter 7. A known amount of PAMAM-ibuprofen conjugate (3 mg/ml) in DDW (1 ml) was also extracted and quantified as the same procedure. The amount of free drug was then calculated from the standard curve.

Chapter Three

Biocompatibility evaluation of branched polymers and dendrimers

3.1. Introduction

Advances in chemical synthesis generates a large number of new water soluble synthetic polymers each year. However, few of these molecules are suitable for use as components of drug delivery systems. An ideal drug-carrier should be non-toxic, non-immunogenic and preferably biodegradable. It should have a high drug carrying-capacity, the ability to deliver drug selectively and also control its rate of release at the target site (reviewed by Duncan 1992; Duncan 1996). All novel polymers must display acceptable biocompatibility and this is one of the most important aspect to prescreen in all polymer candidates proposed for therapeutic use. Acceptable biocompatibility must be established before a new polymer can enter further experiments *in vivo*.

Biocompatibility has been defined by Williams (1986) as "the ability of a material to perform, with an appropriate host response in a specific application". Sgouras & Duncan (1990) proposed a library of biocompatibility tests *in vitro* and *in vivo* as a suitable screening system to evaluate new polymers. *In vitro* biocompatibility tests such as RBC haemolysis and cytotoxicity are used routinely in our laboratory to determine whether new polymers are suitable for further development (Sgouras & Duncan 1990; Carreno-Gomez & Duncan 1997; Malik et al. 1999; Richardson 1999). Other aspects of biocompatibility are also very important such as polymer immunogenicity and biodegradability must also be investigated and these topics have been reviewed elsewhere (Rihova 1996).

Cytotoxicity assays

One of the most important requirements of a polymeric carrier is that it must display no intrinsic cytotoxicity (Sezaki & Hashida 1984). Cell lines have been widely used for *in vitro* screening of the general cytotoxicity of new polymers. The particular cell lines selected would normally be chosen to reflect the proposed polymer application, route of administration, targeted organ and expected organ toxicity (Sgouras & Duncan 1990). Different cell lines display different degrees of sensitivity to toxic agents. In this study two adherent cell lines, B16F10 murine melanoma cells and L132 embryonic lung cells were used to test the general cytotoxicity of different gen of DAE dendrimers. B16F10 cells are easily cultured and they have been widely used previously for testing polymer toxicity both *in vitro* and in *vivo* (Carreno-Gomez & Duncan 1997; Malik et al. 1999; Richardson 1999). Therefore they were chosen as a model to allow comparison of dendrimer toxicity with that of other polymers already described in the literature. The L132 cell lines was used as another model to allow comparison with results obtained with the B16F10 cells.

Many procedures have been used to determine cytoxicity such as [^{3}H]thymidine incorporation (Arnold et al. 1979) and [^{3}H]leucine incorporation (Morgan et al. 1988), counting cell numbers (Arnold et al. 1979), [^{51}Cr]chromium release (Sigot-Luizard & Warocquier-Clerout 1993) and also the measurement of lactate dehydrogenase (LDH) release (Sgouras & Duncan 1990; Sakulnimitr et al. 1995; Macnair et al. 1997). Polymer cytoxicity observed using MTT test was previously shown to give IC_{50} values similar to the values obtained in experiments using [^{3}H]thymidine or [^{3}H]leucine incorporation or counting cell numbers as a measurement of viability (Sgouras & Duncan 1990). In this study the MTT assay was chosen to measure cytotoxicity of DAE polymers. It is a very useful technique for the primary and rapid evaluation of cytotoxicity, and it was originally developed by Mossman to screen new anticancer agents (Mossman 1983). The MTT assay has been shown to be satisfactory for the evaluation of cell viability using several adherent and suspension cell lines (Sgouras & Duncan 1990; Roberts et al. 1996; Zange 1997; Malik et al. 1999).

Everted gut sac viability

The rat everted gut sac has been used over 20 years as an *in vitro* method to measure transport of materials across the gut (Naisbett & Woodley 1994). This system is introduced in more detail in Chapter 4 where the model was selected as an *in vitro* screening system to measure the transfer of radiolabelled PAMAM dendrimers across the gut. It was therefore considered very important to determine whether PAMAM dendrimers would be toxic to intestinal tissue *in vitro*. Here everted rat intestinal sac were incubated with DAE dendrimers and anionic PAMAM dendrimers (gen 2.5, 3.5) and the cationic PAMAM dendrimer gen 4. The ability of the tissue to transport glucose across this tissue by active transport was used as a measure of tissue viability. The glucose concentration inside and outside the everted sac was monitored during the experiment using a modification of the method described by Lloyd & Whelan (1969). As glucose is actively transported, over 2 h viable tissue displays a modest ability to increase in the serosal fluid concentration of glucose compared to that seen in the external medium. Any compound which is toxic to the cells may interfere with the normal active transport process or destroy tight junctions resulting in a loss of ability to maintain the concentration gradient (Pato et al. 1994).

Haemolysis assays

The term "haematocompatibility" can be defined as the possibility of the polymer to induce toxicity by interacting with the soluble components of blood (including proteins and antibodies) and/or cellular components of the blood resulting a disturbance in physiological pathways e.g. blood coagulation system, fibrinolytic system, kinin system, complement system or the immune system (Duncan et al. 1991). Alternatively,

haematocompatibility may be described as the possibility of the blood components themselves to destroy the ability of polymer to perform effectively.

Red blood cell lysis assessment is one simple method that has been used as a preliminary screen for polymer-membrane interaction (Duncan et al. 1991; Duncan et al. 1994) which may have implication for future polymer haematocompatibility *in vivo*. Although following oral administration polymers will not contact blood directly, the RBC lysis assay provides a model to study and quantify the interaction of a potential membrane-active agent and the cellular membrane. Observation made may also provide data supportive to other toxicity measurements e.g. the MTT cytotoxicity assay. During incubation of the polymers with red blood cells, the polymer can interact with cell membrane, and in extreme cases cause membrane disruption haemoglobin release (Duncan et al. 1991). The amount of haemoglobin released in this assay indicates the extent of membrane damage and thus can give a relative index of the toxicity each polymer when compared to the reference materials e.g. dextran as a negative control and PEI as a positive control. To monitor more subtle changes of cell morphology or cell aggregation, SEM has been used.

Polymers and dendrimers

Hyperbranced polymers and dendrimers are new class of polymers. There are very few studies that have investigated systematically their biological properties (review in section 1.5.3). In this study, the biocompatibility of branched polymers (PVF copolymers) and two different classes of dendrimers (DAE and PAMAM dendrimers) have been evaluated.

Many polycations have been examined as potential drug carriers e.g. poly-L-lysine, poly(amidoamine) polymer (Sgouras 1990; Richardson 1999) and more recently polycations have become popular as potential gene delivery system. Poly-L-lysine, chitosan, PEI and cationic dendrimers have all been examined as gene delivery systems (Citro et al. 1994; Tang & Szoka 1997; Ferruti et al. 1998, Richardson 1999). PVF copolymers (Paulus 1996) are new cationic water soluble polymer which were synthesized from polyvinylformamide and partially hydrolyzed with hydrochloric acid (see structure in Figure 2.1). The degree of hydrolysis represents the conversion of polyvinylformamide to polyvinylamine. PVF copolymers contain amino group on the side chain which is suitable for attachment of drug residues or complexation with DNA. They were available in three different Mw range (30 - 1000 kDa) and each Mw range had polymer samples had a different level of substitute with amino groups. Therefore it was considered interesting to investigate the biocompatibility of these polymers before their developing as a drug or gene carrier. Here the haemolytic potential of PVF copolymers

were assessed. The effect of concentration, Mw and side chain amino group content on haemolysis were studied.

Two families of dendrimers were used to study their biocompatibility; DAE dendrimers (Haussling et al. 1998) (Figure 2.2) which contain either a diaminoethane or diaminopropane core and propane (3C) branching unit and PAMAM dendrimer (Figure 2.3) which contain a diaminoethane (ethylenediamine) core and amidoamine branching unit. Both DAE and PAMAM dendrimers are available in different gen and Mw range, and in the case of PAMAM they are also available in different surface charges. Therefore the biocompatibility tests of these dendrimers can be studied systematically. Malik et al. (1999) and Robert et al. (1996) have already studied the biocompatibility of PAMAM dendrimers as introduced in Chapter 1 (section 1.5.4). Consequently, in this study only the haemolytic potential and *in vitro* cytotoxicity of DAE dendrimers were assessed. Polymers which have been used before were adopted as reference controls: poly-L-Lysine, PEI and dextran (Duncan et al. 1996; Sgouras 1990). As this study is interested in the potential development of dendrimers as drug carrier for oral delivery, the toxicity of both DAE and PAMAM dendrimers to intestinal tissue was determined using the everted gut sac viability measurement.

3.2 Methods

Different types of biocompatibility test were carried out as described below.

Haemolysis assay

Nine samples of PVF copolymer of different Mw and amino content (Table 2.1) were assessed for their haemolytic activity using the assay described in section 2.2.2. Dextran (Mw 74,000 Da) and PEI (Mw 70,000 Da) were used as negative and positive reference controls respectively. Incubation times of 1 h and 5 h and a polymer concentration range of 0 - 5 mg/ml were used.

The same RBC lysis assay (section 2.2.2) was also used to determine the haemolytic activity of four gens of DAE dendrimers prepared from either a diaminoethane or a diaminopropane core (Table 2.2). Due to the high degree of haemolysis observed after 1 h, longer incubation times (e.g. 5 h) were not studied. In this case SEM was also used to visualize the dendrimer-RBC interactions. The effect of dendrimers on the pH of PBS was determined over the concentration range used (0 - 5 mg/ml) as described in section 2.2. The effect of pH on RBC stability was studied at 1 h and 5 h.

Cytotoxicity assays

Four gen of DAE dendrimers were examined in respect of their cytotoxicity as described in section 2.1. Prior to the evaluation of the cytotoxicity, the relationship between pH of the medium and dendrimer concentration was also investigated (section 2.1). In order to maintain the same pH of medium, the concentration of dendrimer used in cytotoxicity studies was limited to a maximum of 0.1 mg/ml. Cytotoxicity was determined after incubating dendrimers with B16F10 or L132 cells as described in section 2.1. Dextran (Mw 74,000 Da) and poly-L-Lysine (Mw 56,000 Da) were used as negative and positive reference controls respectively. SEM was also used to substantiate the results generated using this experiment model. IC_{50} values were established by finding the mean (± S.D.) of 3 sets of 8 replicate using linear regression analysis.

Everted gut sac viability

The toxicity of DAE and PAMAM dendrimers towards intestinal tissue was determined using the method described in section 2.3. As DAE 32 was the most toxic in the cell cytotoxicity studies, it was chosen for this study. The effect of DAE 32 on everted gut sac viability was determined over the concentration range 0-0.1 mg/ml using a 30 min incubation (section 2.3).

The cationic PAMAM dendrimer gen 4 and the anionic dendrimers gen 2.5 and gen 3.5 were also assessed for their toxicity towards intestinal tissue using the same procedure (section 2.3). As this study intended to investigate the effect of PAMAM dendrimers on intestinal tissue under the conditions that would be used in the everted sac transport studies (Chapter 4), the longer incubation time of 90 min was used.

3.3. Results

3.3.1. Haemolysis caused by PVF copolymers

All PVF copolymers caused little lysis (generally less than 10%) over the concentration range used after exposure for 1 h (Figure 3.1). In contrast, PEI caused much more lysis above concentration of 0.1 mg/ml and precipitation of haemoglobin at a PEI concentration over 3 mg/ml was observed.

RBC lysis was observed for the 3 different Mw PVF copolymers after a 5 h incubation (Figure 3.2a-c). All PVF copolymers showed greater haemolysis at 5 h than seen at 1 h, and the percentage lysis increased with increasing degree of polymer pendant amine content. Haemolysis also tended to increase with increasing Mw. Lysis was seen to be concentration-dependent only with the PVF copolymer of highest pendant amine content (90% hydrolysis). The PVF copolymers of Mw 30,000 Da were generally poorly

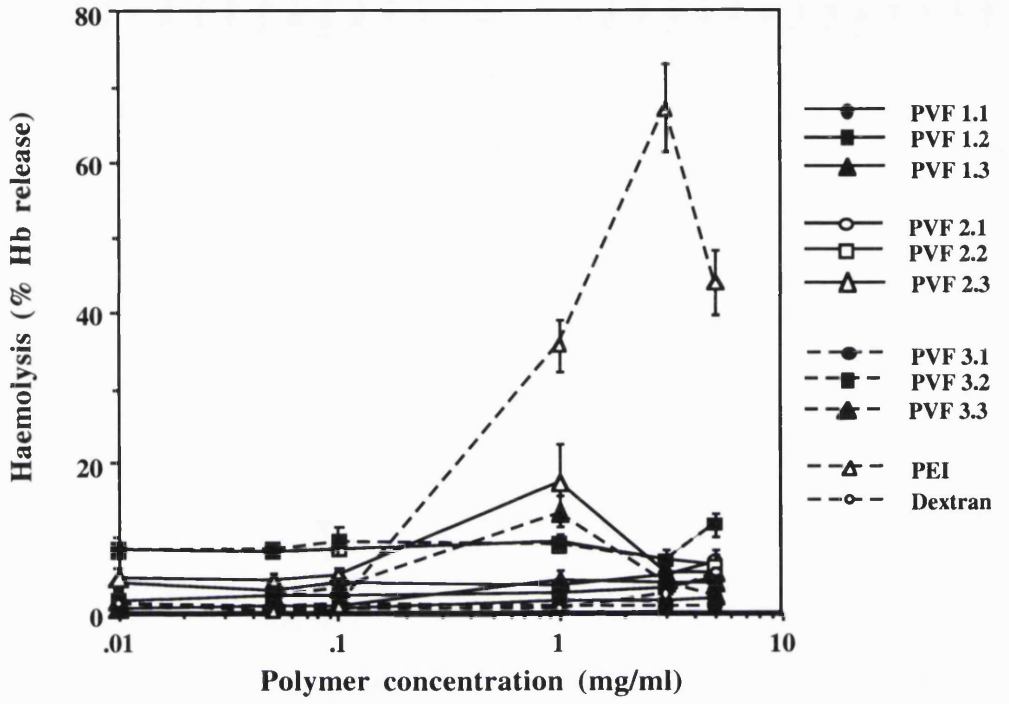


Figure 3.1. Effect of PVF copolymers on rat RBC lysis after 1 h incubation. Data represent the mean ± S.D. (n=24)

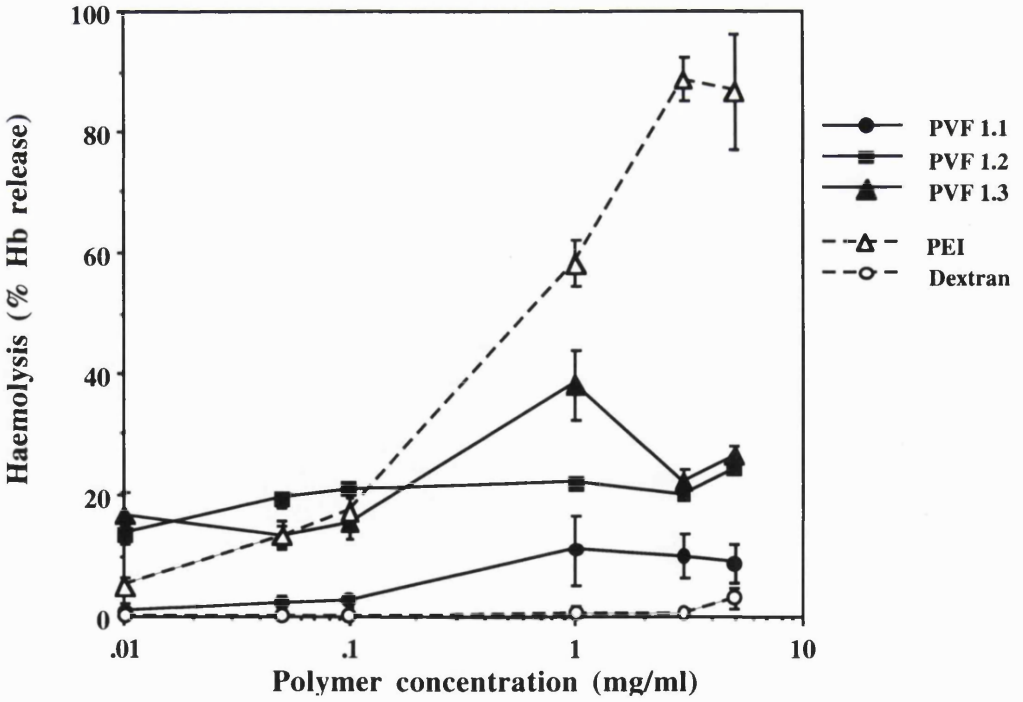


Figure 3.2a. Effect of PVF copolymers Mw 30,000 Da of different amine content on rat RBC lysis after 5 h incubation. Data represent the mean ± S.D. (n=24)

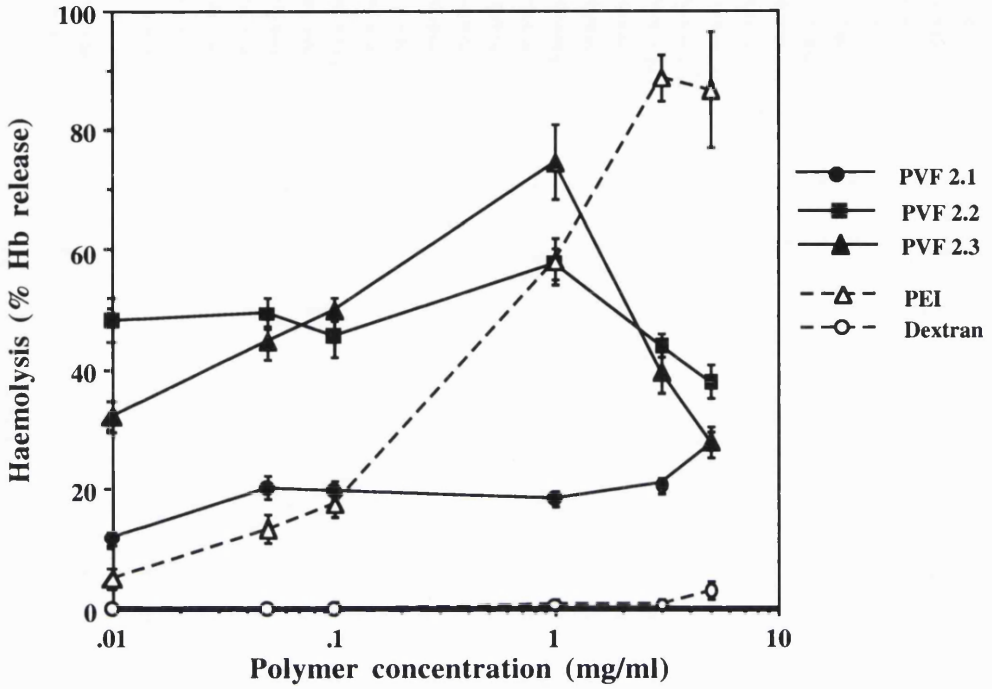


Figure 3.2b. Effect of PVF copolymers Mw 300,000 Da of different amine content on rat RBC lysis after 5 h incubation. Data represent the mean ± S.D. (n=24)

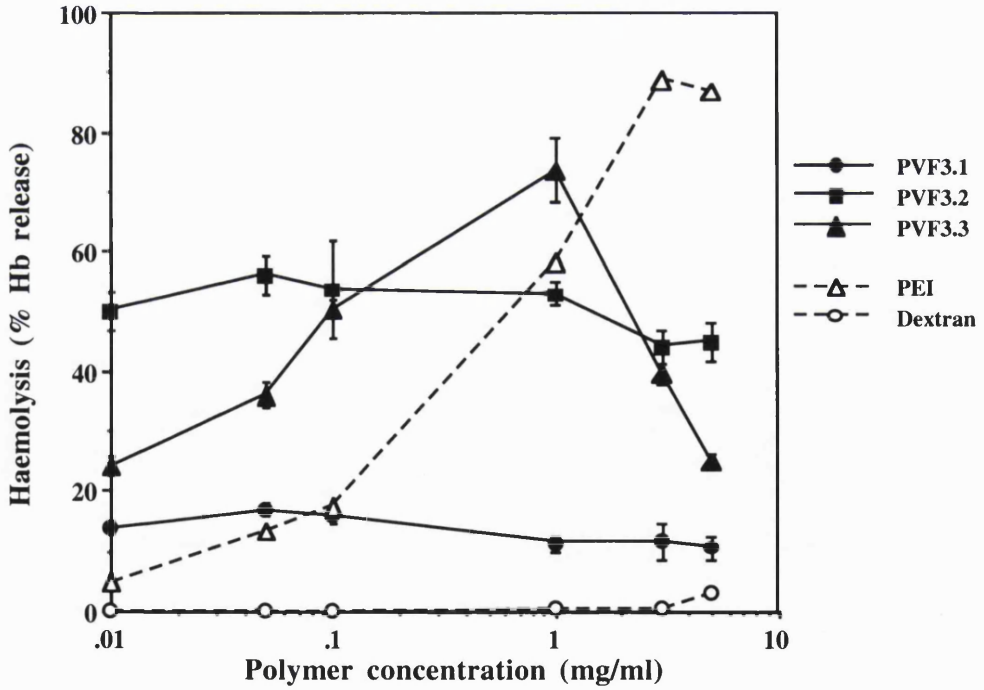


Figure 3.2c. Effect of PVF copolymers Mw 1,000,000 Da of different amine content on rat RBC lysis after 5 h incubation. Data represent the mean ± S.D. (n=24)

haemolytic except PVF 1.3 which has the highest amine group content and this polymer caused lysis above a concentration 0.1mg/ml. It also caused precipitation of haemoglobin at a concentration over 1 mg/ml (Figure 3.2a). The percentage of lysis was significantly higher when RBC were incubated with the higher Mw PVF copolymers (300,000 Da) (Figure 3.2b). PVF 2.1, which has the lowest amine group content, caused approximately 20% lysis, whereas PVF 2.2 and 2.3 caused even more lysis than seen for PEI at all concentrations used. A further increase in the Mw of PVF copolymers up to 1,000,000 Da did not seem to affect the profile of lysis (Fig 3.2c) compared to that seen for copolymers of Mw 300,000 Da. RBC lysis caused by PVF copolymers at a concentration of 1 mg/ml was clearly shown to be related to the degree of hydrolysis (amine group content) and also was Mw-dependent (up to 300,000 Da) (Figure 3.3).

3.3.2. Haemolysis caused by DAE dendrimers

RBC lysis induced by DAE dendrimers after a 1h incubation is shown in Figure 3.4. It can be seen that all the DAE dendrimers were lytic above a concentration of 1 mg/ml in a concentration-dependent manner. Throughout the lysis observed was less than seen for PEI over the concentration range used, except in the case of the DAE dendrimer with 32 surface groups which showed the same degree of lysis as PEI (3 mg/ml). There was no significant difference in the pattern of lysis seen for the DAE dendrimers except for DAE 32 which showed greater haemolysis than seen for the other gens at concentration 3 and 5 mg/ml (Figure 3.4).

Observation of RBC morphology using SEM showed changes in RBC appearance that were consistent with the more quantitative results obtained in the haemolysis study (Figure 3.5). PEI, the reference polymer, caused substantial membrane damage at a concentration of 1 mg/ml. RBCs exposed to DAE 32 at a concentration of 1 mg/ml showed a total loss of cell morphology whilst RBCs exposed to the same dendrimer at concentration 0.01 mg/ml showed no change in cell appearance.

As DAE dendrimers are very basic in character (Table 2.2), it was considered important to determine their effect on the pH of PBS. It can be seen from Figure 3.6 that the pH was markly increased with increasing concentration of dendrimer added. The pH of the solution was approximately 8 at a dendrimer concentration of 0.1 mg/ml, but this increased to pH~ 10.5 and 11 at dendrimer concentrations of 1 and 5 mg/ml respectively. Consequently, the effect of pH and incubation time on RBC stability was also studied (Figure 3.7). Least haemolysis was observed at pH 7.4 although little increase occured between pH 5.5 and pH 9 observed. However, at pH 11 the percent of haemolysis observed was almost 100%. The incubation time did not have effect on the extent of lysis observed.

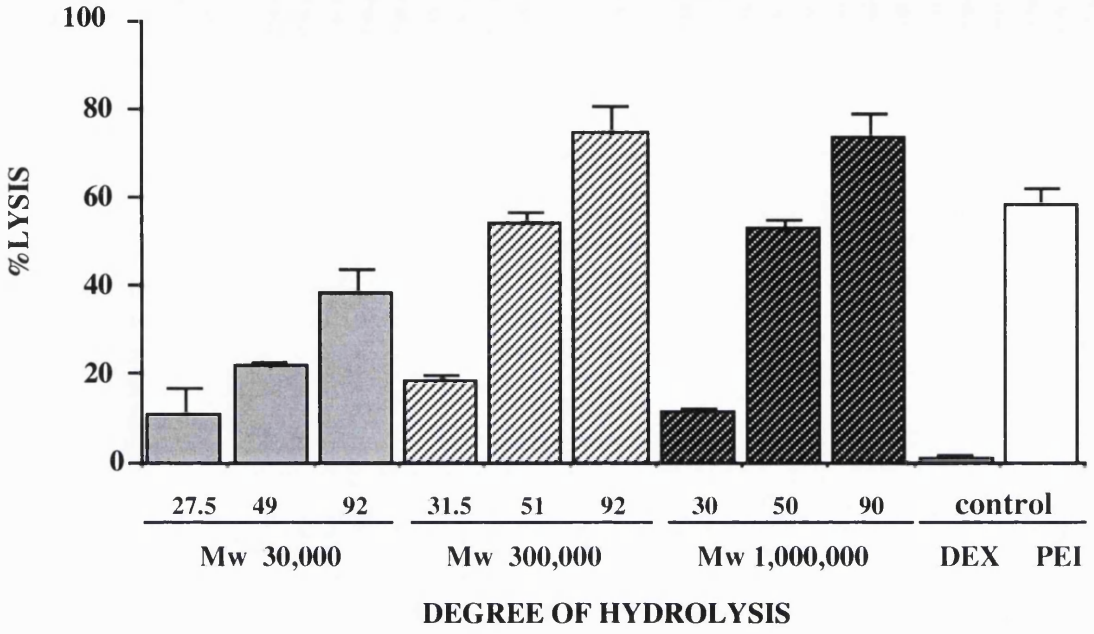


Figure 3.3. Effect of Mw and degree of hydrolysis (amine group content) of PVF copolymers on RBCs lysis at 1mg/ml (5h incubation)

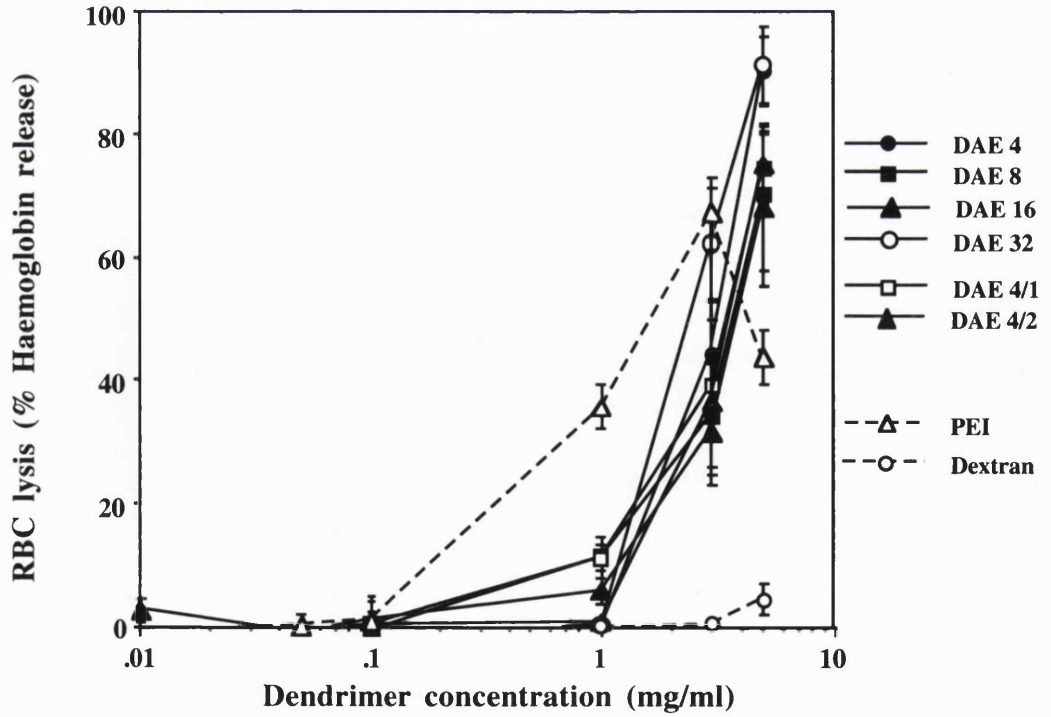
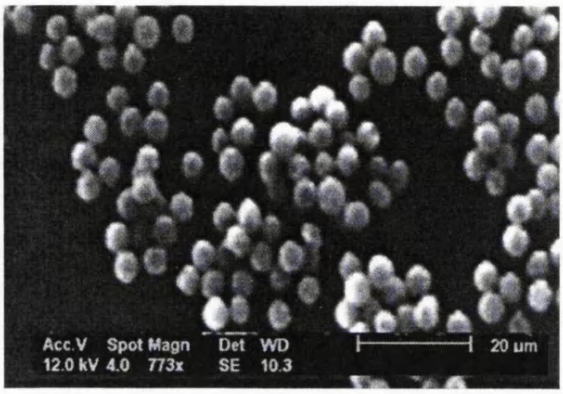
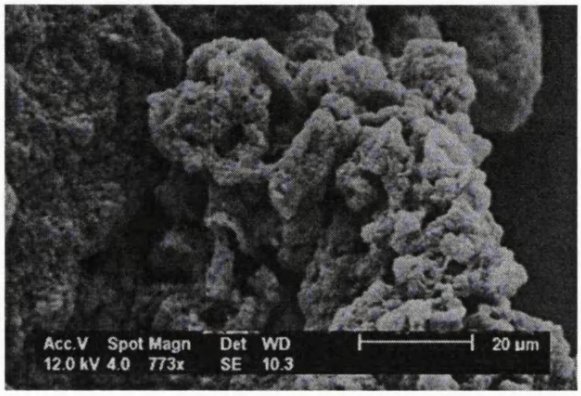


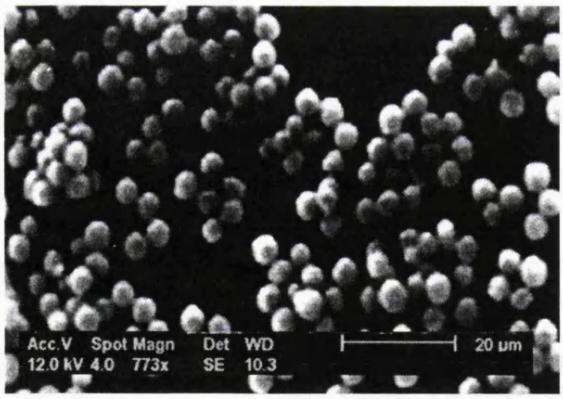
Figure 3.4. Effect of DAE dendrimers on rat RBC lysis after 1 h incubation. Data represent the mean ± S.D. (n=24)



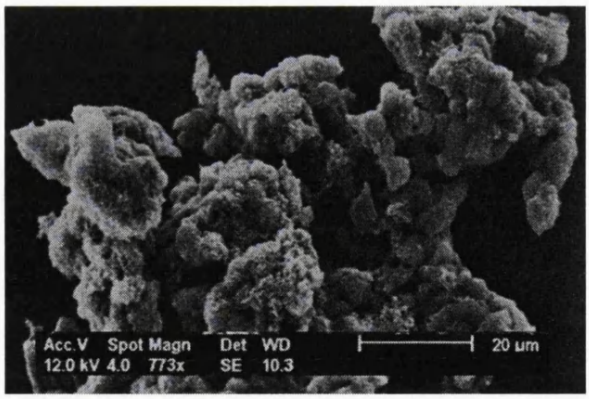
PBS Control



PEI Control
(1 mg/ml)



DAE 32
(0.01 mg/ml)



DAE 32
(1 mg/ml)

Figure 3.5. RBC morphology after incubation with DAE 32 dendrimers or PEI for 1 h

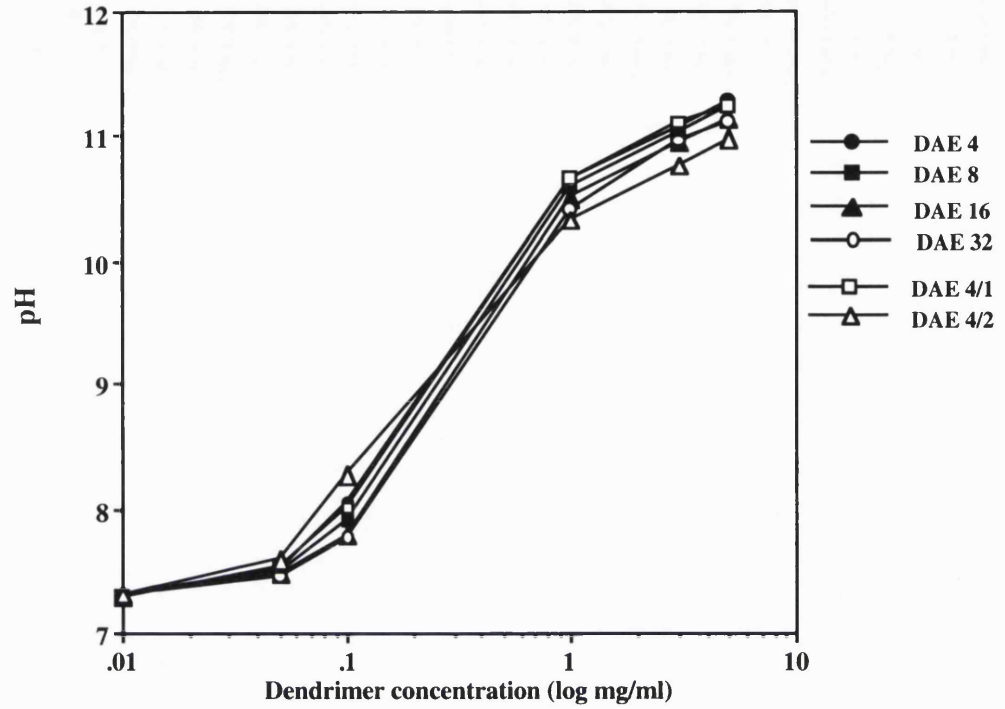


Figure 3.6. Effect of DAE dendrimer concentration on pH of PBS. Data represent the mean ± S.D. (n=3).

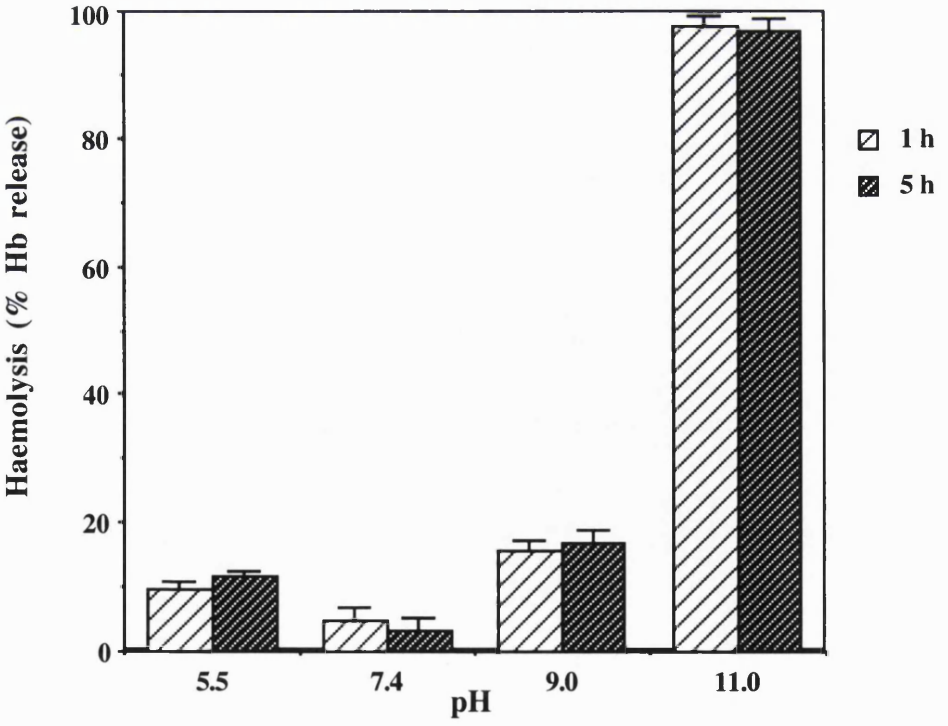


Figure 3.7. Effect of pH on RBC lysis after incubation at 1 and 5 h. Data represent the mean ± S.D. (n=8).

3.3.3. In vitro cytotoxicity of DAE dendrimers towards B16F10 and L132

A change in pH of the tissue culture medium was observed at DAE dendrimer concentrations above 0.1 mg/ml (Figure 3.8). Thus in order to maintain physiological medium pH, the maximum concentration of dendrimer used was limited to 0.1 mg/ml. The viability of B16F10 and L132 cells exposed to DAE dendrimers is shown in Figure 3.9 and 3.10 respectively. The cytotoxicity observed against both cell lines was gen and concentration-dependent. The IC_{50} values of DAE dendrimers measured for B16F10 and L132 cells are shown in Table 3.1. After a 72 h incubation, only DAE 32 caused a decrease in viability of B16F10 cells at concentration above 0.01 mg/ml and the IC_{50} value was 0.05 ± 0.01 mg/ml.

SEM indicated changes in the morphology of B16F10 cells after exposure to DAE 32 that was consistent with the decrease in viability measured by the MTT test (Figure 3.11). In the case of the L132 cell line, both DAE 16 and DAE 32 were cytotoxic whilst the other lower generations were not. The IC_{50} value measured for DAE 16 against L132 cells was 0.03 ± 0.01 mg/ml, whereas the IC_{50} values seen for DAE 32 and poly-L-lysine were identical at 0.01 ± 0.01 mg/ml.

3.3.4. Toxicity towards everted intestinal tissue

The effect of DAE 32 and PAMAM dendrimers gen 2.5, 3.5 and 4 on the viability of everted rat intesinal tissue was studied *in vitro*. After incubation with DAE 32 for 30 min the glucose concentration measured inside the sac (in the serosal fluid) was significantly higher than the concentration measured outside (in the culture medium) for all dendrimer concentrations except at the highest concentration used (0.1 mg/ml). In this case the intestinal tissue showed inability to maintain the glucose concentration gradient (Figure 3.12). The effect of DAE 32 on rat everted intestinal tissue was consistent with the cytotoxicity observed against B16F10 and L132 cells.

A similar trend was observed for cationic PAMAM dendrimer (Figure 3.13). Everted intestinal sacs were capable of maintaining the glucose concentration gradient in the presence of PAMAM gen 4 except at the highest concentration of 0.1 mg/ml. The anionic PAMAM dendrimers (gen 2.5 and gen 3.5) did not affect glucose accumulation at all the concentrations used (Figure 3.14).

3.4. Discussion

The PVF copolymers studied here caused little RBC lysis at 1 h compared to PEI but they were haemolytic after 5 h. Within each Mw range, haemolysis increased as a function of amine content, e.g. PVF which contained 90% amine groups was more lytic

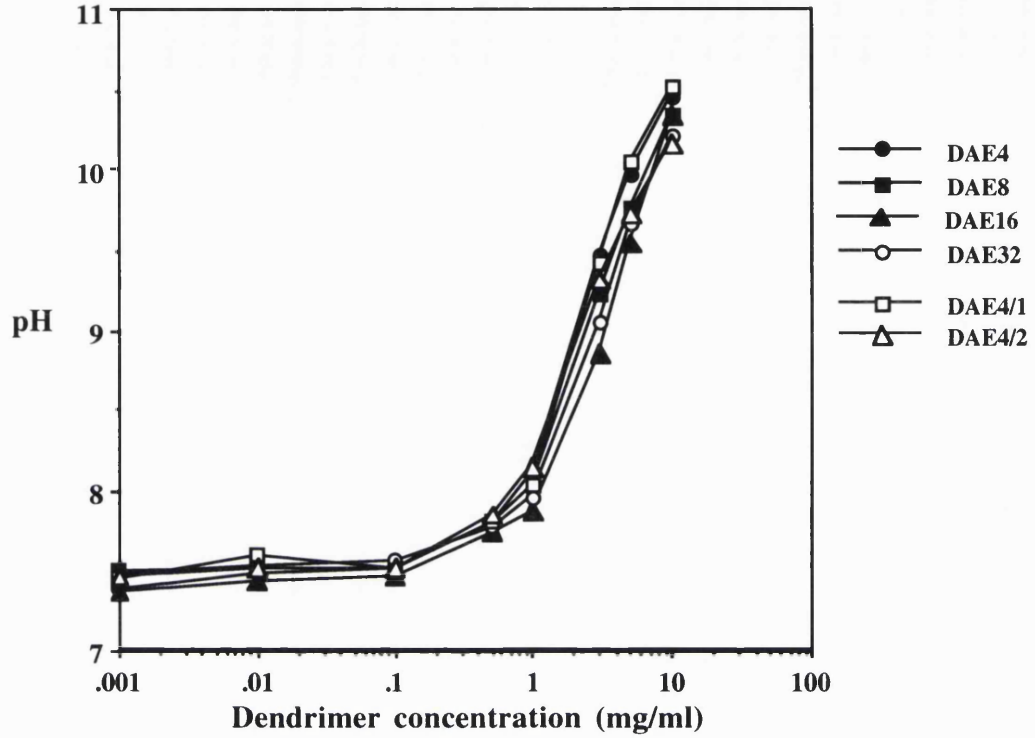


Figure 3.8. Effect of DAE dendrimer concentration on pH of RPMI 1640 medium. Data represent the mean ± S.D. (n=3).

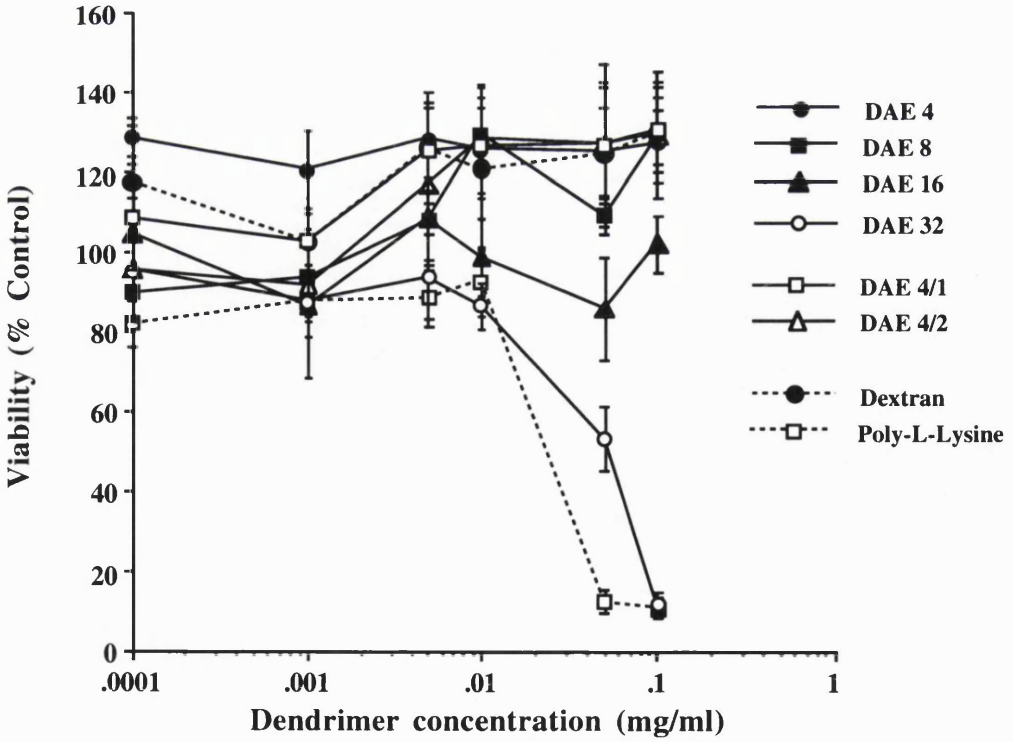


Figure 3.9. Effect of DAE dendrimer on the viability of B16F10 cells. Data represent the mean ± S.D. (n=24).

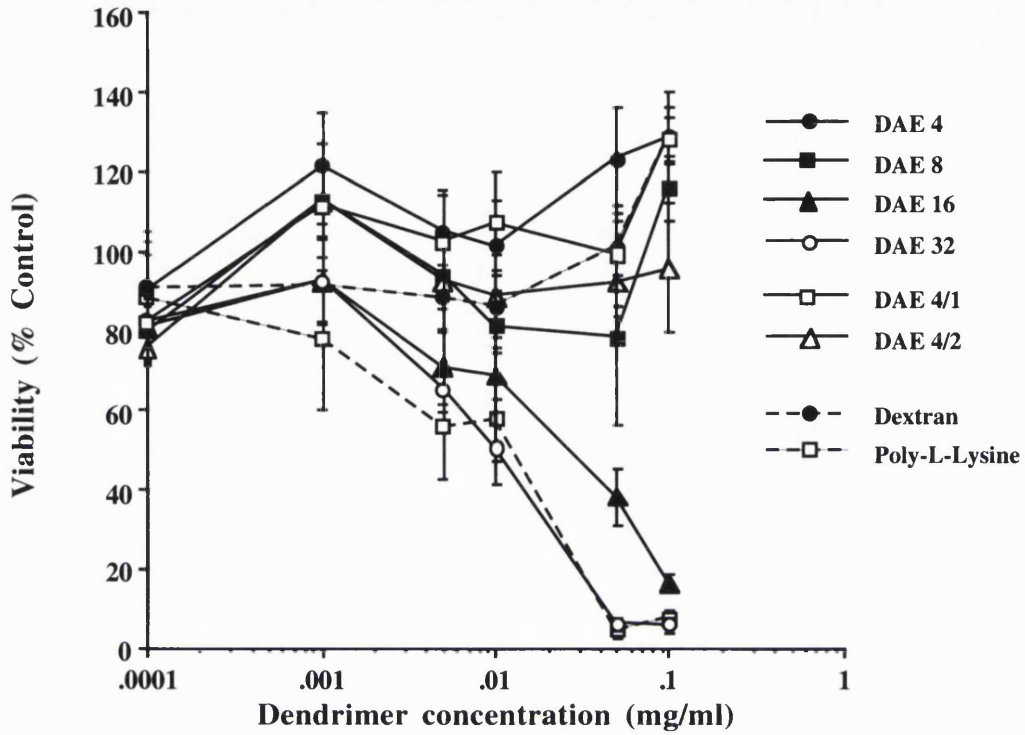
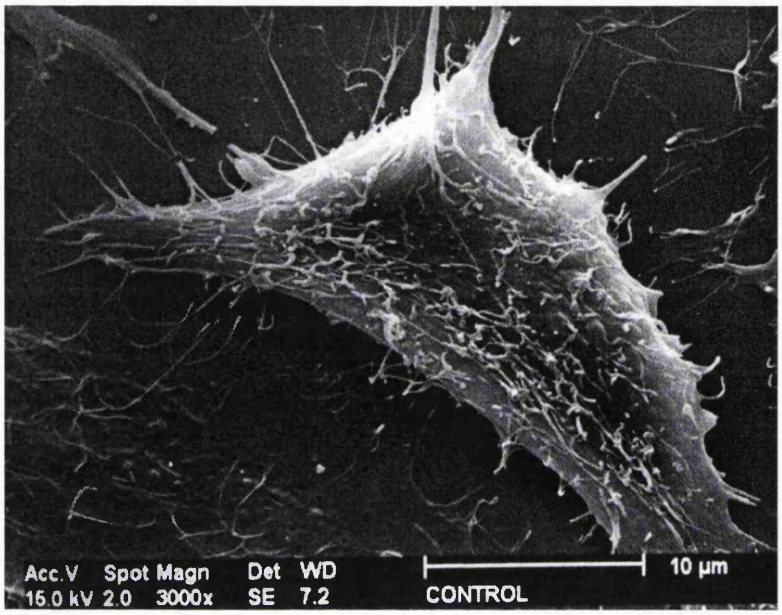


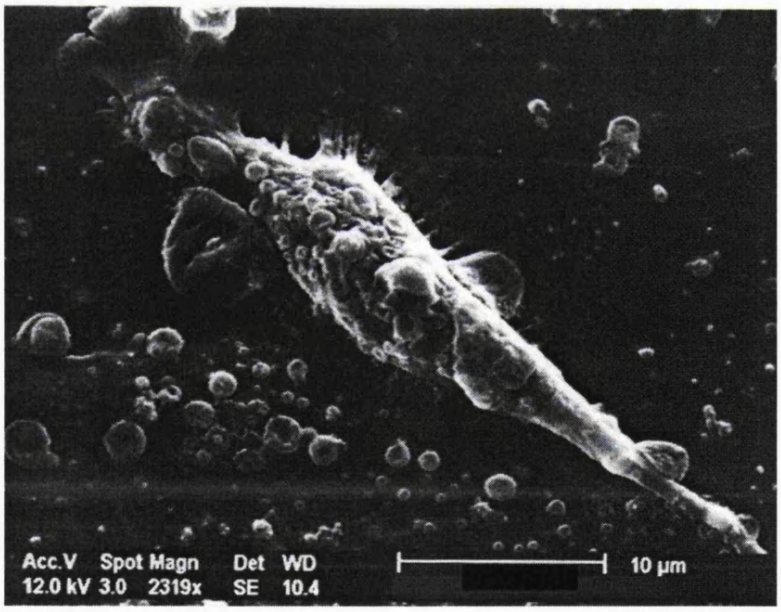
Figure 3.10 Effect of DAE dendrimer on the viability of L132 cells. Data represent the mean ± S.D. (n=24).

Table.3.1. IC_{50} values (mg/ml) measured for DAE dendrimers in two different cell lines. Data represent the mean of 3 experiments (n = 24) ± S.D.

Dendrimer	B16F10	L132
DAE 4	>0.1	>0.1
DAE 8	>0.1	>0.1
DAE 16	>0.1	0.03±0.01
DAE 32	0.05±0.01	0.01±0.00
DAE 4/1	>0.1	>0.1
DAE 4/2	>0.1	>0.1
Poly-L-Lysine	0.04±0.00	0.01±0.01
Dextran	>0.1	>0.1



PBS Control



DAE 32

Figure 3.11. SEM of B16F10 cells exposed to DAE 32 dendrimers (0.01 mg/ml) for 1 h

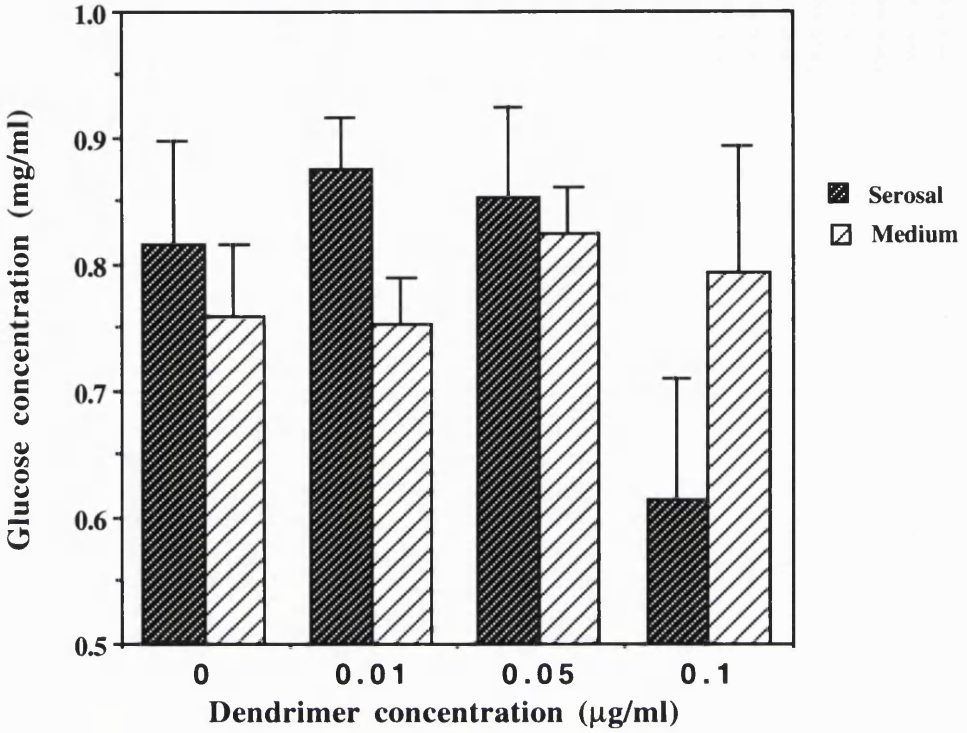


Figure 3.12. Effect of DAE concentration on the accumulation of glucose in the serosal fluid. Data represent the mean ± S.D. (n=9).

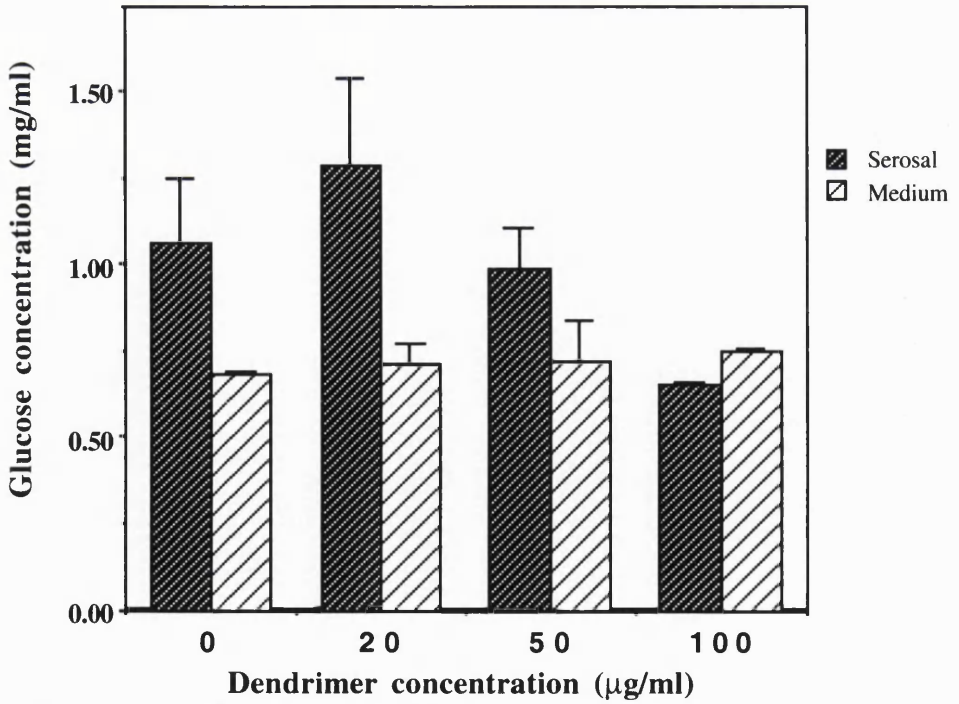
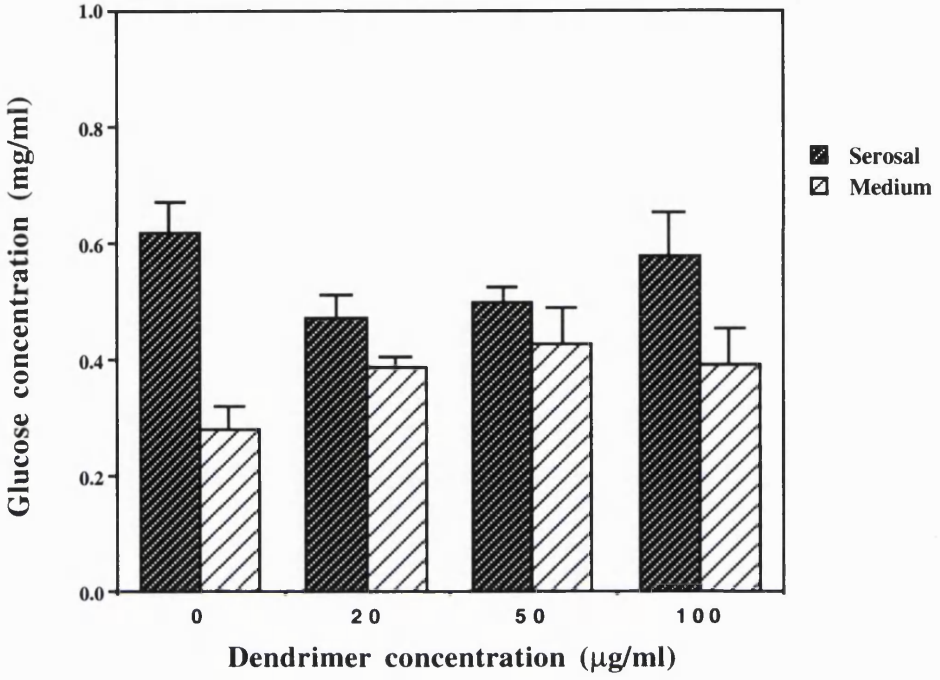


Figure 3.13. Effect ofPAMAM gen 4 concentration on the accumulation of glucose in the serosal fluid. Data represent the mean ± S.D. (n=9).

(a)



(b)

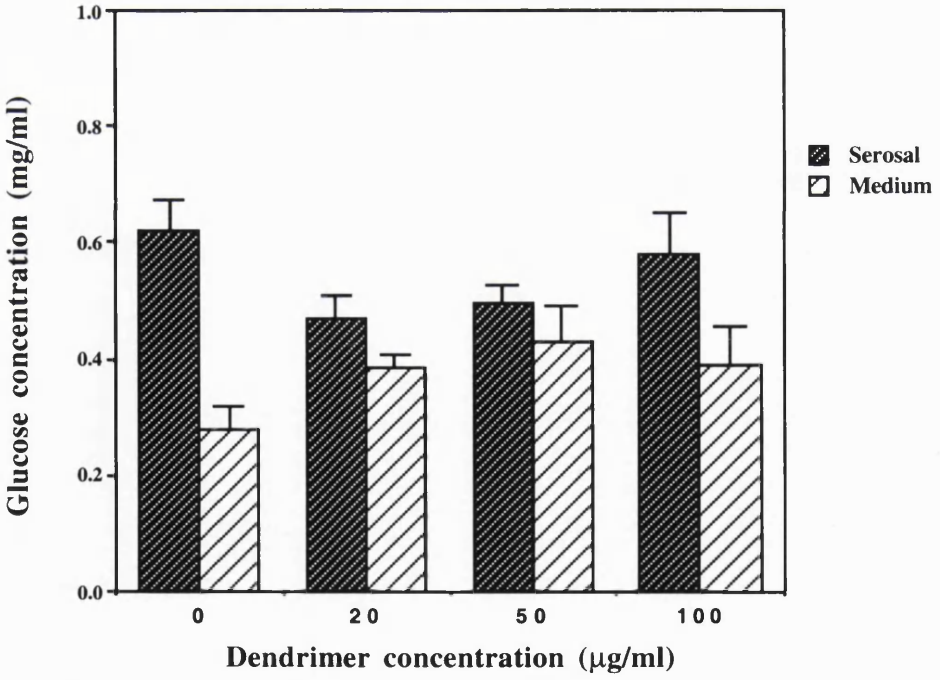


Figure 3.14. Effect of anionic PAMAM concentration on the accumulation of glucose in the serosal fluid. Panel (a) gen 2.5 and Panel (b) gen 3.5
Data represent the mean ± S.D. (n=9).

than PVF which contained 30% amine groups. These results are consistent with other studies which showed that the cationic amine groups of polymers such as poly-L-lysine, PEI, poly(amidoamine)s, and chitosan were responsible for the lysis of RBC (Sgouras & Duncan 1990; Carreno-Gomez & Duncan 1997; Richardson 1999). Table 3.2 shows the concentration of PVF copolymers and other cationic polymers that cause > 20 % lysis at 1 h and 5 h. PVF copolymers and other cationic polymers were not haemolytic up to the maximum concentration used after 1 h incubation except chitosan hydroglutamate and the poly(amidoamine) ISA 4 which caused lysis above concentration 1 mg/ml and 4.5 mg/ml, respectively. Although there are no data available at 5 h incubation for the other polymers, some PVF polymers such as PVF 2.2, 2.3, 3.2 and 3.3 with high amine content caused greater haemolysis than PEI. Polycations probably caused haemolysis because of electrostatic interaction of protonated amino groups of the polymer with the anionic charge of the erythrocyte membrane (Nevo et al. 1955). This interaction would cause sheer stress and rupture to cell membrane leading to haemoglobin release.

Haemolysis of PVF copolymers was Mw-dependent after 5 h exposure indicating that the larger cationic polymers were generally more effective at interacting with the RBC membrane. However, as PVF Mw was increased from 300,000 to 1,000,000 Da haemolysis did not increase indicating a size limit to the Mw effect (Figure 3.2b-3.2c). As PVF copolymers are linear polymers, there would be the optimum size range of molecules that can interact competently with the erythrocyte membrane. Haemolytic activity of PVF copolymers was concentration-dependent only for the samples which had a high content of amine groups (PVF 1.3, 2.3 and 3.3). This shows the importance of polymer charge density.

RBC lysis studies showed that cationic DAE dendrimers, regardless of gen and core composition, were lytic above concentrations of 1 mg/ml (Figure 3.5). This could again be attributable to the cationic nature of these hyperbranched molecules. These observations are consistent with haemolysis studies that have been reported using other cationic dendrimers (Table 3.3). DAB (gen 2-4) and PAMAM (gen 4) dendrimers which bear amine terminal groups were also lytic above a concentration of 1 mg/ml whereas anionic dendrimers such as PAMAM dendrimers bearing carboxylate terminal groups were not haemolytic up to the maximum concentration used (5 mg/ml). It is interesting that both DAE and DAB families were equally lytic and the lysis were not gen-dependent. On the other hand, PAMAM dendrimers-induced haemolysis was clearly gen-dependent. This is predictable owing to the similarity of the repeat branch structure (-$NCH_2CH_2CH_2N$-) of DAE and DAB dendrimers and the difference in the branch structure of PAMAM dendrimers (-$NCH_2CH_2CONHCH_2CH_2N$-). However, other

Table 3.2. The effect of PVF copolymers on haemolysis compared with other cationic polymers

Polymers	Concentration (mg/ml)*		References
	1 h incubation	5 h incubation	
PVF 1.1	no lysis	no lysis	This study
PVF 1.2	no lysis	no lysis	
PVF 1.3	no lysis	> 0.1	
PVF 2.1	no lysis	> 3	
PVF 2.2	no lysis	> 0.01	
PVF 2.3	no lysis	> 0.01	
PVF 3.1	no lysis	no lysis	
PVF 3.2	no lysis	> 0.01	
PVF 3.3	no lysis	> 0.01	
PEI	> 0.1	> 0.1	This study
Poly-L-Lysine	no lysis	Not tested	Richardson 1999
Poly(amidoamine) ISA 1, 9	no lysis	Not tested	Richardson 1999
Poly(amidoamine) ISA 4	> 4.5	Not tested	Richardson 1999
Chitosan hydrochloride	no lysis	Not tested	Carreno-Gomez & Duncan 1997
Chitosan hydrolactate	no lysis	Not tested	Carreno-Gomez & Duncan 1997
Chitosan hydroglutamate	> 1	Not tested	Carreno-Gomez & Duncan 1997

* Concentration which caused haemolysis more than 20 % (The maximum concentration used for all experiment was 5 mg/ml except chitosan (3 mg/ml)

Table 3.3. The effect of DAE dendrimers on haemolysis and viability of B16F10 cells compared with other cationic and anionic dendrimers (Malik et al. 1999)

Dendrimers	Number of surface groups	Termini	Concentration* (mg/ml) which caused lysis at 1 h incubation	IC_{50}
DAE				
gen 0	4	$-NH_2$	> 1	0.1
gen 1	8	$-NH_2$	> 1	0.1
gen 2	16	$-NH_2$	> 1	0.1
gen 3	32	$-NH_2$	> 1	0.05 ± 0.01
DAB				
gen 2	16	$-NH_2$	> 1	0.3
gen 3	32	$-NH_2$	> 1	0.05
gen 4	64	$-NH_2$	> 1	0.05
gen 1.5	16	COOH	no lysis	no toxic
gen 2.5	32	COOH	no lysis	no toxic
gen 3.5	64	COOH	no lysis	no toxic
PAMAM				
gen 1	8	$-NH_2$	no lysis	no toxic**
gen 3	32	$-NH_2$	> 4	0.1
gen 4	64	$-NH_2$	> 1	0.1
gen 1.5	16	COONa	no lysis	no toxic**
gen 2.5	32	COONa	no lysis	no toxic**
gen 3.5	64	COONa	no lysis	no toxic**
Poly-L-Lysine				0.04 ± 0.01

* Concentration which caused haemolysis more than 20 % (The maximum concentration used for all experiment was 5 mg/ml)
**The maximum concentration used was 2 mg/ml

factors may contribute to the haemolysis observed for example the Mw and pH of each dendrimer type studied.

The cytotoxicity of DAE dendrimers towards B16F10 and L132 cells revealed that the high gens were toxic in a concentration-dependent manner. A comparison of cytotoxicity of DAE dendrimers with that seen for other families of dendrimers against B16F10 cells is summarised in Table 3.3. The high gen cationic PAMAM dendrimers (gen 3 and gen 4) and DAB dendrimers (gen 2-4) are markly cytotoxic and displayed IC_{50} values similar to those seen for poly-L-lysine (Malik et al. 1999). PAMAM dendrimers of equivalent surface functionality were slightly less toxic than DAB and DAE dendrimers with the same number of surface groups. In contrast, anionic dendrimers were not cytotoxic towards B16F10 cells up to maximum concentration used (2 mg/ml) (Malik et al. 1999). Another study showed that the cytotoxicity of cationic PAMAM (gen 3, gen 5 and gen 7) against V79 Chinese hamster lung fibroblast was concentration- and generation-dependent (Roberts et al. 1996). According to these results, the L132 cell line seemed to be more sensitive to the effect of DAE dendrimer than B16F10 cells. Although the IC_{50} values observed for poly-L-Lysine in both cell lines were not significantly different, the value for DAE 32 against B16F10 (0.05 ± 0.01 mg/ml) was higher than seen against L132 cells (0.01 ± 0 mg/ml). In addition, DAE 16 was shown to be toxic only towards L132 cells.

Consistent with the cytotoxicity study, DAE 32 showed concentration-dependent effect. At the highest concentration (0.1 mg/ml) it was toxic to intestinal tissue and resulted on impaired ability of the tissue to accumulate glucose into serosal fluid. The toxicity of PAMAM dendrimers towards intestinal tissue confirmed previous studies which report the haemolytic behaviour and cytotoxicity of cationic PAMAM dendrimers. Conversely, anionic PAMAM dendrimers were neither lytic nor cytotoxic over the broad concentration range used and they showed no evidence of toxicity *in vivo* after repeated i.p. (95 mg/kg) injection to mice (Malik et al. 1999).

The results of these biocompatibility studies demonstrate the importance of early biological testing in determining the potential of novel polymers for *in vivo* use. Consistently, the polymers that have cationic nature due to protonated amine groups in their structures showed toxicity dependant on concentration, Mw (gen) and exposure time.

PVF copolymers with low Mw and lower amine content were shown to be safer for systemic administration compared to those with higher Mw and higher amine content. Further experiments are needed to establish their full biocompatibility profile including

biodistribution and immunogenicity studies. The DAE dendrimers used in this study may not be suitable for *in vivo* use since they were haemolytic after a 1h incubation. Also the higher gens were toxic to cell lines and intestinal tissue. The results from previous studies and this study showed that PAMAM dendrimers with cationic surface were toxic to RBC, cell lines and intestinal tissue. In contrast, anionic PAMAM dendrimers were much less toxic and show potential as safe drug carriers for both i.v. and oral administration. Therefore, PAMAM dendrimers were selected for further evaluation as oral drug delivery systems.

Chapter Four

Uptake of ^{125}I -labelled PAMAM dendrimers by the rat small intestine in vitro

4.1. Introduction

As PAMAM dendrimers with an anionic surface were shown to be biocompatible *in vitro* (results from chapter 3 and Malik et al. 1999), and they have shown potential as targetable carriers for the anticancer agent cisplatin (Malik 1999), it was considered interesting to study their potential as a novel system for oral drug delivery. In the context of oral drug delivery, biocompatible dendrimers could be useful in many applications for example 1) Increase solubility of poorly soluble drugs, 2) Control the rate of release, 3) Control GI transit of drugs by acting as new oral bioadhesive, 4) Promote transfer across the GI epithelium and 5) Target to specific regions in the GI tract and/or target to specific organs following oral absorption. These concepts were described in Chapter 1.

The nanoscale, particularly spherical, architecture of dendrimers suggested that they might have potential to transfer across the mucosal barrier so it was decided to first investigate their ability to transverse the GI mucosa. Several *in vitro* models have been used to determine the rate and mechanism of intestinal absorption of drugs and potential drug carriers. Widely used systems include the Ussing chamber containing *ex vivo* tissue slices, cultured cells and the everted gut sac model. These *in vitro* screening systems are described briefly here.

Ussing chamber

The Ussing chamber was first introduced by Ussing and Zerahn (1951) for measuring ion transport across frog skin. Later this became commonly used for the study of drug absorption across intestinal tissue. The system consists of the small section of intestine which is clamped between two chambers, both containing buffers (reviewed by Barth et al. 1999). The compound of interest is added to the donor compartment and its accumulation in the reciever compartment is measured as a function of time. The compound can be exposed to either the mucosal or serosal surface thus allowing the measure of directional-fluxes mucosal to serosal or serosal to mucosal. Evaluation of these parameters can help to characterise passive versus carrier-mediated transport (Stewart et al. 1997). This model is useful for measuring drug transport across tissues derived from specific intestine sites e.g. duodenum, colon and is also useful for the evaluation of drug metabolism. The Ussing chamber allows measurement of the electrical properties of the mucosa during the experiment, thus tissue viability and integrity can be monitored by measuring short circuit current and transepithelial resistance. However, a major weakness of this model is the fact that most of studies have used simple salt buffers as an incubation medium, often with a long incubation period. According to Levine et al. (1970) and Plumb et al. (1987) studies, rapid tissue damage occurs after incubating GI tissues in these simple saltsolutions for longer than 20-30 min. Another disadvantage of the Ussing chamber model is that the surface area of the tissue used is relatively small

compared with the volume of the receiving chamber so that the dilution and sensitivity of measurements can be a problem unless it is overcome by using radiolabelled compounds (reviewed by Barth et al. 1999).

Cultured cells

In recent years, cultured cell monolayers have become widely used as a model to study intestinal drug absorption. From a basic research perspective, these systems offer the potential to easily manipulate the environment and/or cellular properties allowing study of the mechanism of drug absorption. From a drug discovery perspective, these monolayer systems can be automated and used to identify compounds with favorable pharmacokinetic properties, and to evaluate structure-absorption/metabolism relationship and toxicity of compounds (Quaroni & Hochman 1996). The Caco-2 cell monolayer is the most extensively used cell monolayer model. These cells are derived from a human colon carcinoma (Artursson et al. 1996). Caco-2 cells differentiate spontaneously to enterocytes under conventional cell culture conditions upon reaching confluence on porous support, and thus resemble the small intestinal epithelium. (Gan & Thakker 1997). The Caco-2 model enables both apical to baso-lateral and baso-lateral to apical transport to be studied, with the apical compartment mimicking the intestinal lumen and the basal compartment mimicking the bloodstream (reviewed by Barth et al. 1999). The major advantage of Caco-2 model is that the cells are human in origin so there is no problem concerning the interspecies differences. In addition, the method does not require the use of animals, unlike most *in vitro* models. However, there are a number of limitations: (1) Caco-2 cells are a cancer-derived line and therefore may have properties different from normal cells, (2) the monolayer lacks mucin-producting globet cells, and thus the effect of the mucus layer cannot be evaluated, (3) the cell monolayer preparation time is long (2-4 weeks), (4) a very slow transport rate is observed and (5) variable quantitative results have been obtained in different laboratories due to viability in the culture and experimental conditions (Walter & Kissel 1995). To overcome the problem of lack of mucus secretion, the HT29 cell line has recently been introduced (Karlsson et al. 1993).

The everted sac system

The everted intestinal sac system was first used by Wilson & Wiseman (1954) to study the transport of sugars and amino acids. The technique consists of everting a freshly excised rat small intestine, filling it with medium (early studies used simple salt solution) at 37°C, dividing into sacs (approximately 2-4 cm in length) using braided surgical silk. Each sac is then incubated with a given substrate to study the uptake into epithelial tissue by assaying material in the cells and study the transfer across the epithelial layer by measuring material that found inside the sac at the end of incubation period. In

the late 1970's, Woodley and colleagues (Naisbett & Woodley 1994) developed this system further by using the complex tissue culture medium (TC199) (instead of simple salt buffer) to ensure tissue integrity and morphology. It should emphasised that use of tissue culture medium ensures tissue viability and metabolic activity for extended incubation times up to 2 h and this is a considerable improvement on the basic technique (Bridges et al. 1978; Bridges et al. 1980; Barth et al. 1998). The viability of the intestinal tissue can be proven by measuring the transport of glucose from the tissue culture medium into the serosal fluid against the concentration gradient.

The everted gut sac technique affords a simple, rapid, reproducible, inexpensive and reliable model for screening for drug absorpion and its enhancement (Barth et al. 1999). It has been used to study the uptake of liposomes and proteins (Rowland & Woodley 1981a-c), macromolecules with potential for use in oral drug delivery systems (Cartridge et al. 1986; Blundell et al. 1993; Pato et al. 1994) and microspheres (Carreno-Gomez 1999). It can also be used to identify mechanisms of absorption (Naisbett & Woodley 1994), preferential sites of absorption as well as evaluating the role of p-glycoprotein (Barth et al. 1998).

Therefore for this initial study it was decided to use the rat everted sac system (Naisbett & Woodley 1994) to study the uptake into intestinal tissue and translocation across the intestinal mucosa of ^{125}I-labelled PAMAM dendrimer both anionic and cationic surface (in the non-toxic concentration used (Chapter 2)). The effect of dendrimer generation, surface charge and concentration on the uptake and transport were also studied. To quantify the amount of material either in the tissue or serosal fluid, a sensitive technique is required as the amount of tissue for any one sac is small. Radioisotopes afford the most sensitive method and have been used to study the transport of several macromolecules across the intestinal tissue (Cartridge et al. 1986; Blundell et al. 1993; Naisbett & Woodley 1994; Pato et al. 1994). Therefore, the ^{125}I-labelled PAMAM dendrimer were prepared and used in this study.

4.2. Methods

Radioionation and characterisation of PAMAM Dendrimers

PAMAM dendrimers having either a carboxylate surface (gen 2.5, gen 3.5 and gen 5.5) or an amine surface (gen 3 and gen 4) were radioiodinated (section 2.2.6) and the specific activity and the amount of unbound [^{125}I]iodide were determined by low voltage paper electrophoresis (section 2.2.7).

Uptake and serosal transport of ^{125}I-labelled PAMAM dendrimers by the rat small intestine in vitro

The uptake and serosal transport of PAMAM dendrimers was quantified by incubating everted intestinal sacs with ^{125}I-labelled dendrimer as described in section 2.2.8. A dendrimer concentration of 20 μg/ml was selected for the initial experiments as it was shown to be non toxic in the glucose active-transport assay (Chapter 3). The effect of dendrimer gen and surface functionality on tissue uptake and serosal transport was determined over 120 min. In addition the concentration-dependency (0-100 μg/ml) of both processes was studied using a 60 min incubation time. All uptake results are expressed in terms of the amount of dendrimer taken up per unit tissue protein (ng / mg tissue protein) and the rates of uptake are expressed in terms of EI (Willaims et al. 1975) as described in section 2.2.8. The amount of tissue protein was determined by a modified Lowry protein assay (section 2.2.8). The nature of the radioactivity measured in the medium and serosal fluid was determined by sephadex G-25 column chromatography (section 2.2.8). Intestinal tissue viability was confirmed by measuring the concentration of glucose in the serosal fluid and incubation medium (section 2.2.8) and expressing the results as a medium to serosal ratio.

Uptake and serosal transport of free [^{125}I] iodide by the rat small intestine in vitro

The uptake and transfer of free [^{125}I]iodide was studied over 120 min as described in section 2.2.8. Effect of concentration of free [^{125}I]iodide on the accumulation in tissue and serosal was also determined after 1 h incubation (section 2.2.8).

4.3. Results

4.3.1. Characterisation of PAMAM Dendrimers

The labelling effiencies of carboxylate and amine dendrimers were in the range of 90-95% and 75-81%, respectively (Table 4.1). Unbound [^{125}I]iodide in each preparation was less than 1%. Examples of the paper electrophoresis patterns observed before and after purification are shown in Appendix I.

4.3.2.Uptake and transfer of ^{125}I-labelled PAMAM dendrimer with time

Tissue uptake and serosal transfer of ^{125}I-labelled PAMAM dendrimers with anionic and cationic surface are shown in Figure 4.1-4.2, respectively. It can be seen that the tissue and serosal accumulation of all anionic dendrimers (Figure 4.1a-b) increased linearly with time, thus enabling EIs to be calculated by regression analysis. The mean EIs obtained are shown in Table 4.2. The rates of serosal transfer were higher than the rates measured for tissue uptake for all anionic dendrimers. The rate of tissue uptake of

Table 4.1. Labelling efficiency and purity of ^{125}I-labelled PAMAM dendrimers

Dendrimer	Labelling efficiency (% bound vs free)	Final purity (% free ^{125}I)	Specific activity (μCi/mg)
Carboxylate			
Gen 2.5	90.7	0.73	1.1
Gen 3.5	94.4	0.84	1.2
Gen 5.5	94.1	0.31	2.1
Amine			
Gen 3	75.6	0.71	9.4
Gen 4	80.5	0.98	11.8

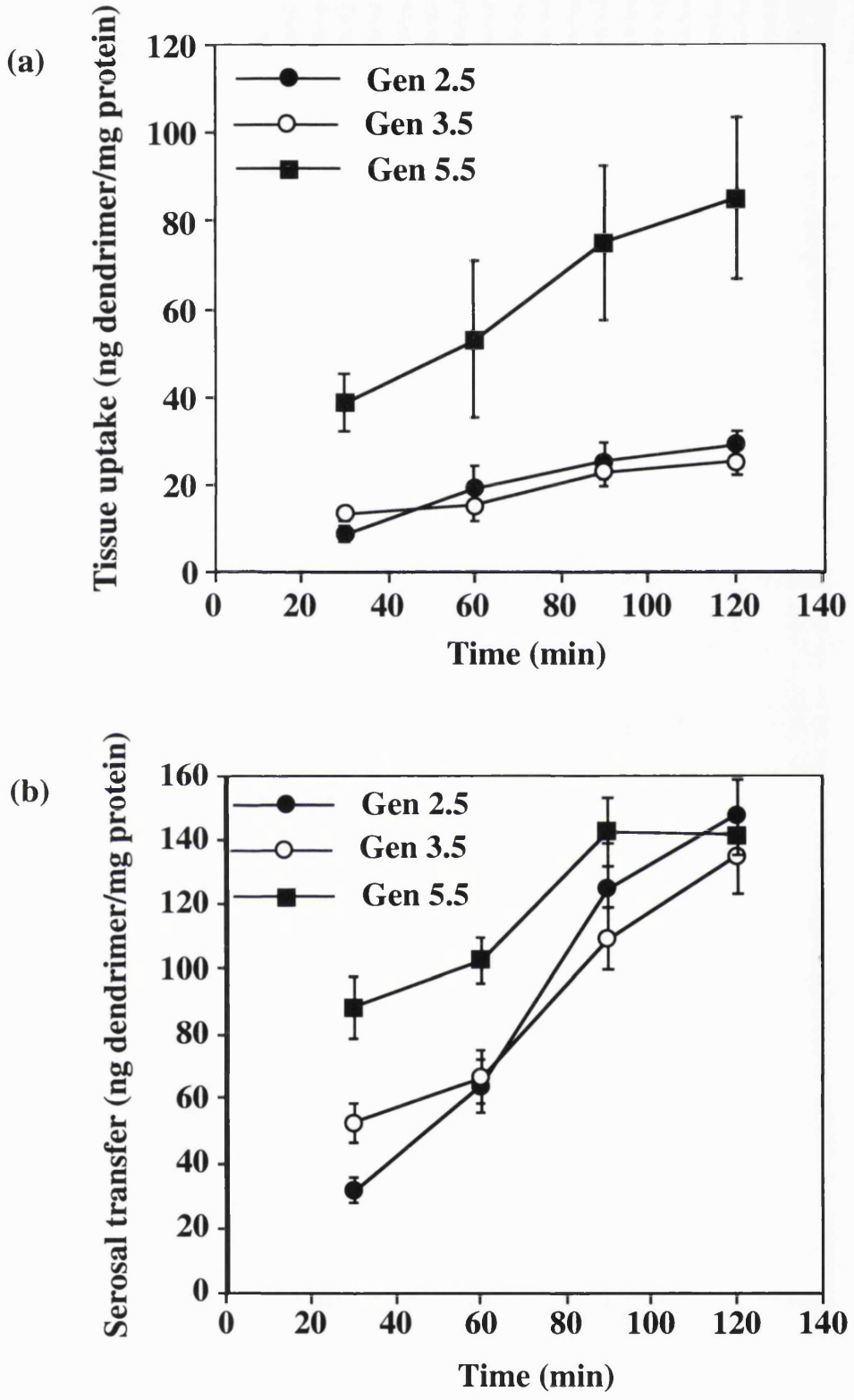


Figure 4.1. Tissue uptake and serosal transfer of ^{125}I-labelled anionic PAMAM dendrimers. Panel (a) shows the tissue uptake with time and panel (b) the serosal transfer with time ; mean (n = 9) ± S.D.

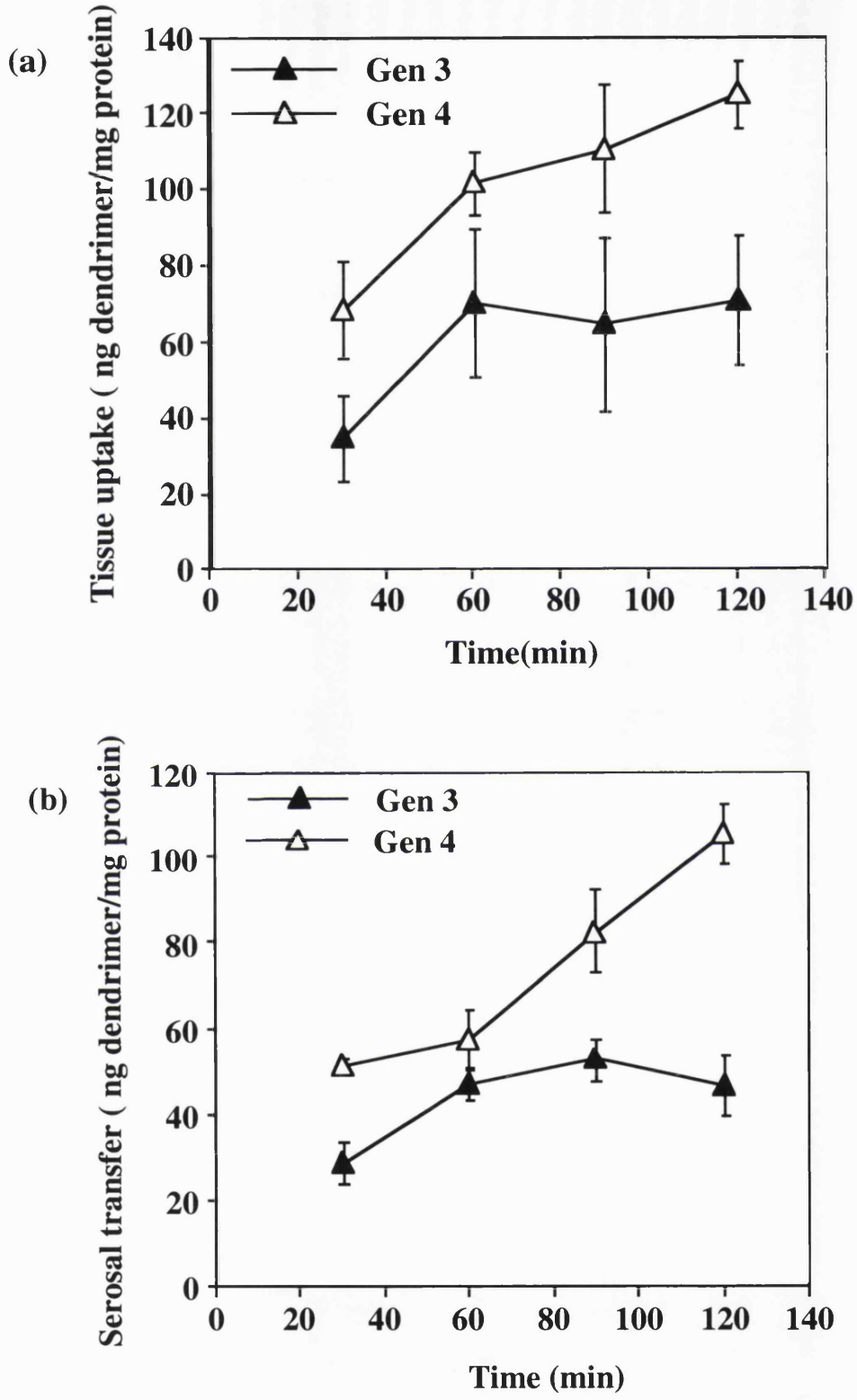


Figure 4.2. Tissue uptake and serosal transfer of ^{125}I-labelled cationic PAMAM dendrimers. Panel (a) shows the tissue uptake with time and panel (b) the serosal transfer with time ; mean (n = 9) ± S.D.

Table 4.2. Endocytic indices of ^{125}I-labelled PAMAM dendrimers

Dendrimer	Endocytic index (μl/mg protein/h)	
	Tissue	Serosal
Gen 2.5	0.76 ± 0.07	4.04 ± 0.99
Gen 3.5	0.65 ± 0.09	3.39 ± 0.51
Gen 5.5	2.48 ± 0.51	4.40 ± 0.67
Gen 3	3.33 ± 0.86	2.34 ± 0.28
Gen 4	3.46 ± 0.65	2.45 ± 0.37

^{125}I-labelled gen 5.5 was considerably higher (2.48 ± 0.51 µl/mg protein/h) than seen for ^{125}I-labelled gen 2.5 and 3.5 (0.6-0.7 µl/mg protein/h). In contrast, the serosal transfer rates for all anionic PAMAM dendrimers were similar and the EI values were high (3.4-4.4 µl/mg protein/h). Approximately 15-20% of the radioactivity associated with gen 2.5 and 3.5 was recovered in the tissue whereas 80-85% was transferred to the serosal fluid. In the case of gen 5.5, approximately 30-35% of the radioactivity was recovered in the tissue and 65-70% transferred to the serosal fluid (Table 4.3).

The ^{125}I-labelled cationic PAMAM dendrimers showed a different pattern of accumulation (Figure 4.2). The tissue uptake was usually higher than serosal transfer at each time point. Over the first 60 min, both gen 3 and 4 displayed a similar rate of tissue uptake (EI = 3.3-4.8 µl/mg protein/h) and serosal transfer (EI = 2.3-2.7 µl/mg protein/h). After 60 min, both the tissue and serosal accumulation of gen 3 plateaued, whereas gen 4 showed slower rate of tissue uptake and constant rate of serosal transfer (Figure 4.2). Throughout approximately 55-60% of the radioactivity associated with gen 3 and 4 was located in the tissue and only 35-40% of the radioactivity in the serosal fluid (Table 4.3).

4.3.3. Effect of ^{125}I-labelled PAMAM dendrimer concentration on the uptake

The effect of PAMAM dendrimers concentration on the tissue uptake and serosal transfer are shown in Figure 4.3. The tissue and serosal accumulation of anionic dendrimers increased with increasing substrate concentration. At the same concentration, gen 5.5 showed a higher amount of both radioactivity in the tissue and serosal fluid than seen for gen 2.5 and gen 3.5. For the anionic dendrimers (all concentrations), about 30-35% of the ^{125}I-labelled gen 5.5 was detected in the tissue and 65-70% was found in the serosal fluid, approximately 15-20% of ^{125}I-labelled gen 2.5 and ^{125}I-labelled gen 3.5 were recovered in the tissue whereas 80-85% of the radioactivity was recovered in the serosal fluid (Table 4.4).

The uptake of cationic dendrimers also increased with increasing concentration (Figure 4.3). For example, in the case of ^{125}I-labelled gen 4 the tissue levels were approximately 55-60% at most dendrimer concentrations but at concentration 100 µg/ml, the tissue levels increased up to 80% (Table 4.4).

4.3.4. Analysis of the serosal contents and culture medium by gel permeation chromatography

The elution profiles of all dendrimers in serosal fluid collected after 2 h and the parent ^{125}I-labelled PAMAM dendrimers are shown in Figures 4.4-4.5. The elution

Table 4.3. Percentage of radioactivity recovered in tissue and serosal fluid

Time (min)	% of radioactivity recovered									
	gen 2.5		gen 3.5		gen 5.5		gen 3		gen 4	
	serosal	tissue	serosal	tissue	serosal	tissue	serosal	tissue	serosal	tissue
30	78.5	21.5	80.0	20.0	69.0	31.0	45.3	54.7	42.7	57.3
60	77.2	22.8	81.6	18.4	65.2	34.8	40.0	60.0	36.0	64.0
90	83.2	16.8	82.7	17.3	64.4	35.6	44.8	55.2	42.6	57.4
120	83.6	16.4	84.3	15.7	61.8	38.2	39.7	60.3	45.7	54.3

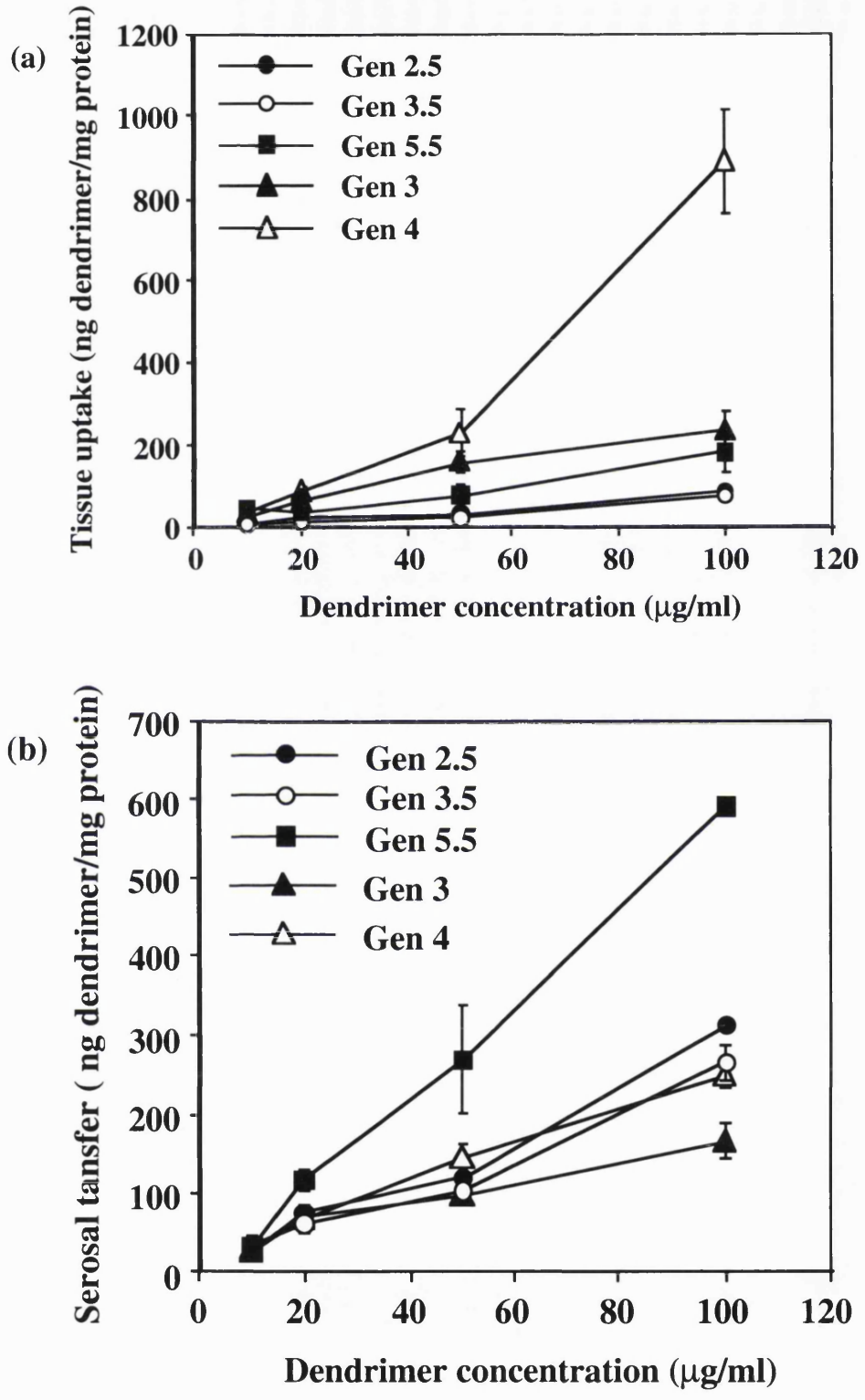


Figure 4.3. Effect of dendrimer concentration on tissue uptake (panel a) and serosal transfer (panel b) at 60 min; mean (n = 9) ± S.D.

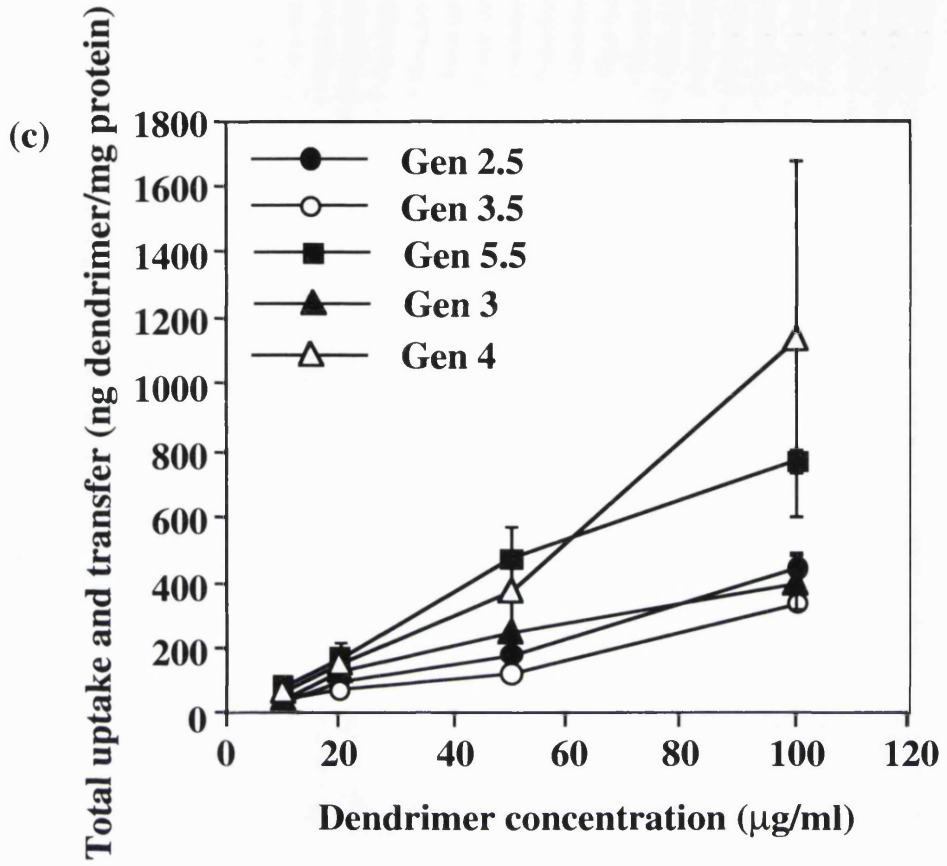


Figure 4.3. (continued) Effect of dendrimer concentration total uptake (panel c) at 60 min; mean (n = 9) ± SD.

Table 4.4. Percentage of radioactivity recovered in tissue and serosal fluid at different concentration of dendrimer

Concentration (μg/ml)	% of radioactivity recovered									
	gen 2.5		gen 3.5		gen 5.5		gen 3		gen 4	
	serosal	tissue	serosal	tissue	serosal	tissue	serosal	tissue	serosal	tissue
10	80.2	19.8	89.0	11.0	65.5	34.5	59.7	40.3	45.3	54.7
20	81.2	18.8	87.1	12.9	66.2	33.8	52.8	47.2	43.8	56.3
50	77.6	22.4	84.5	15.5	73.2	26.8	38.9	61.2	38.6	61.4
100	77.4	22.6	79.1	20.9	70.3	29.7	41.5	58.5	21.9	78.1

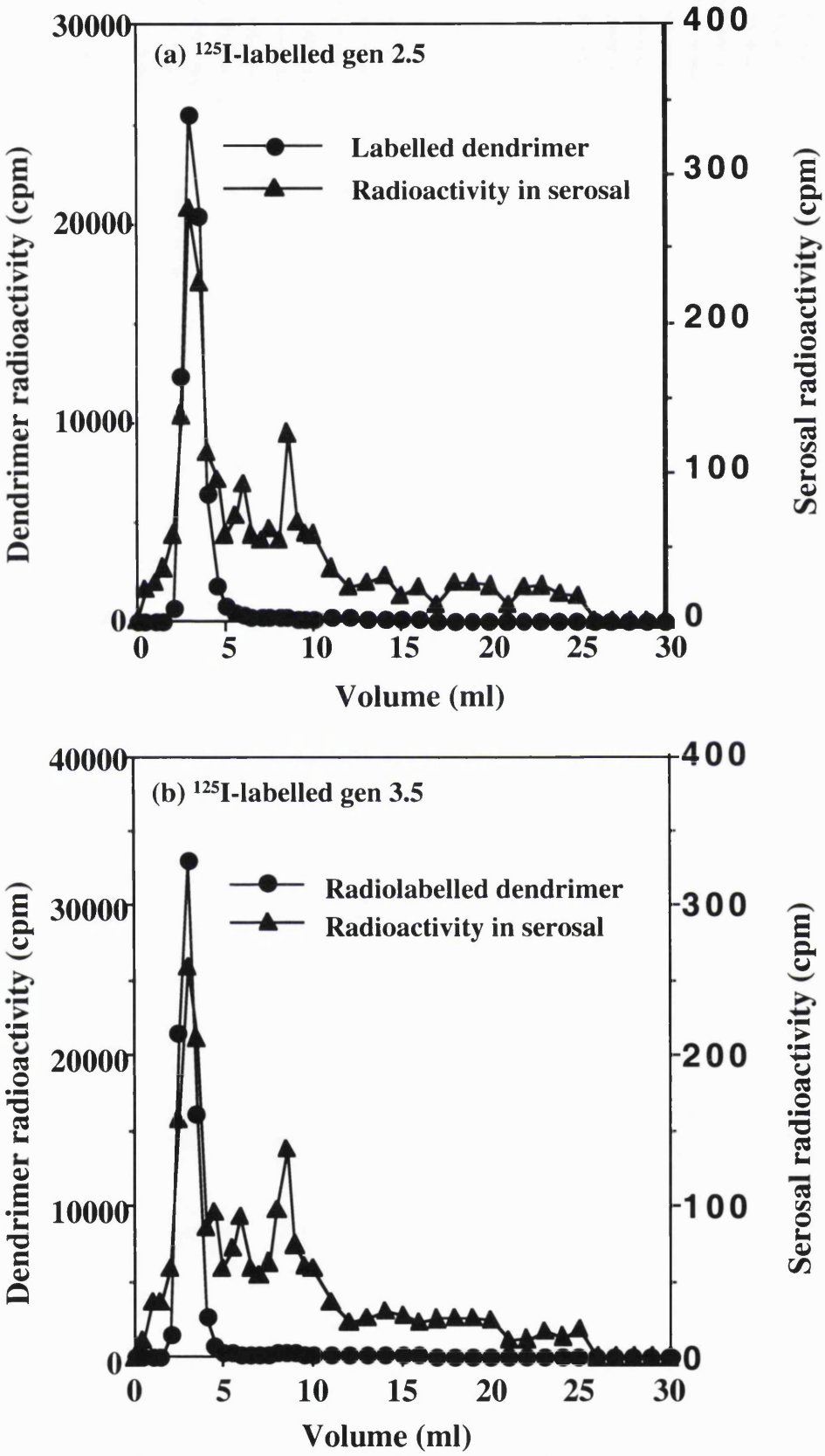


Figure 4.4. Elution profile obtained by GPC of the radioactivity in the serosal fluid of sacs incubated for 2 h in the presence of anionic dendrimer compared with the original profile of radiolabelled dendrimer

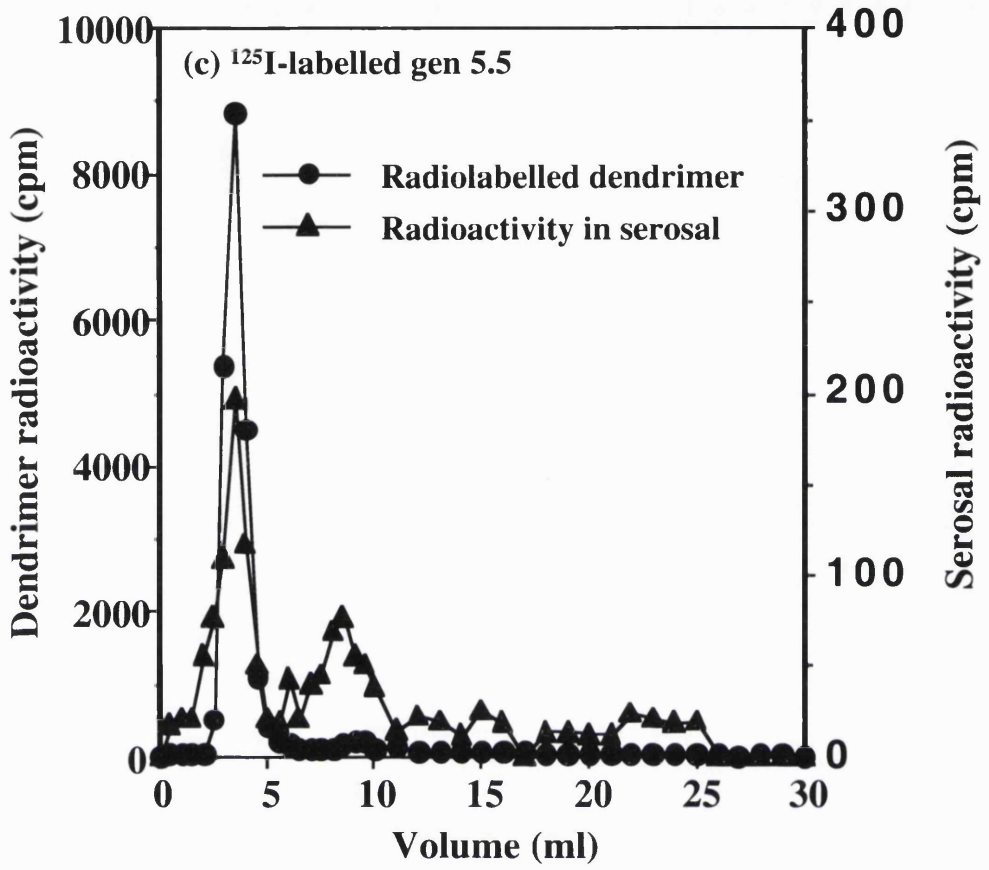


Figure 4.4. (continued) Elution profile obtained by GPC of the radioactivity in the serosal fluid of sacs incubated for 2 h in the presence of anionic dendrimer compared with the original profile of radiolabelled dendrimer

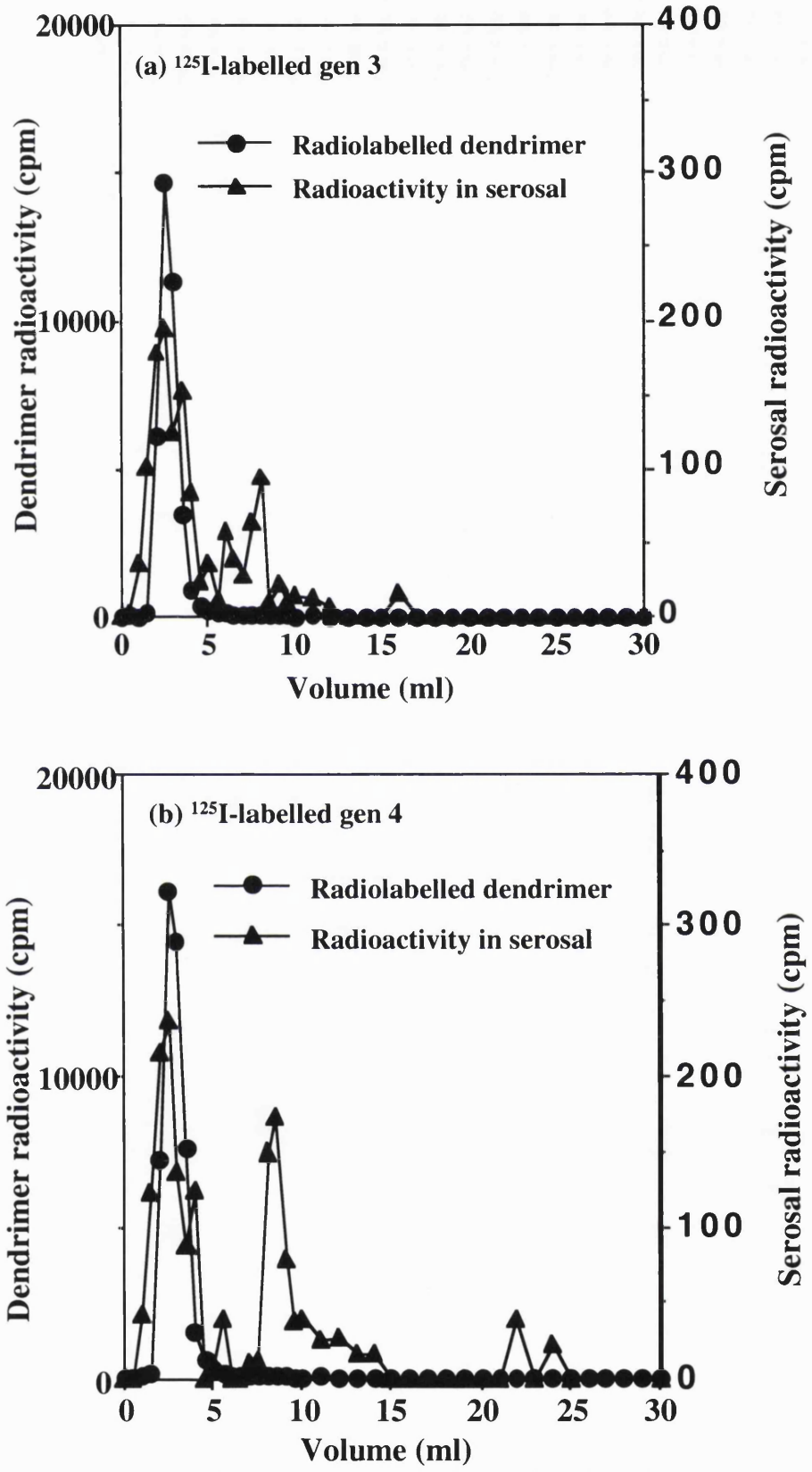


Figure 4.5. Elution profile obtained by GPC of the radioactivity in the serosal fluid of sacs incubated for 2 h in the presence of cationic dendrimer compared with the original profile of radiolabelled dendrimer

profiles obtained indicated radioactivity in both high and low Mw fractions. The low Mw fraction represented in each case was 30-38%. The calculated percentage of low Mw ^{125}I-labelled product in serosal fluid and also in the medium after 2 h incubation were calculated from the area under the curve and shown in Table 4.5. There was no direct correlation between the amount of low Mw ^{125}I-labelled product in the medium and the serosal fluid.

4.3.5. Uptake and transfer of sodium [^{125}I]iodide

The characteristics of tissue uptake and serosal transfer of free [^{125}I]iodide are shown in Figure 4.6. The results indicated that serosal transfer of free [^{125}I]iodide tended to be saturated at the early incubation times and that progressive tissue accumulation did not occur. The uptake and transfer of free [^{125}I]iodide at different concentrations is shown in Figure 4.7. The transfer increased linearly with free [^{125}I]iodide concentration in medium. Approximately, 96-100 % of the radioactivity recovered was found in the serosal fluid and only 0- 4 % in the tissue.

4.3.6. The viability of rat everted intestinal sacs

Throughout the viability and integrity of the sacs was monitored to confirm that the PAMAM dendrimers did not damage the intestinal tissue and that serosal uptake was not due to simply tissue leakage. As glucose is actively transported, there was an increase concentration inside the sacs (serosal side) compared with the medium outside (mucosal side), and hence the concentration gradient. The ratio of glucose concentration (medium: serosal) after incubating with ^{125}I-labelled PAMAM dendrimers for 60 and 120 min are shown in Table 4.6. There was a higher concentration of glucose in the serosal fluid than found in the external medium, and the concentration ratio medium: serosal increased progressively with time. This indicated that the sacs were intact and viable throughout the time course of experiments. In addition, active transport of H^+ ions by the sacs could also be observed. As the tissue culture medium contains a coloured indicator (phenol red) for visual estimation of medium pH. During the incubation, the medium inside the sac changed colour from red to yellow whereas the medium outside remained original red colour.

4.4. Discussion

Neutral hydrophilic polymers of Mw 10,000 to 40,000 Da such as ^{125}I-labelled polyvinylpyrrolidone (PVP), ^{125}I-labelled poly (N-vinylpyrollidone-co-maleic anhydride) (NVPMA) (Pato et al. 1994) and ^{125}I-labelled N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers (Cartridge et al. 1986) were shown to be taken up by everted intestinal sacs by fluid-phase endocytosis and transported across relatively slowly. Typically they display EI values in the range 0.6 - 1.6 µl/mg protein/h for tissue uptake

Table 4.5. Calculated percentage of low Mw ^{125}I-labelled product found in medium and serosal fluid after 2 h incubation

Dendrimer	% low Mw ^{125}I-labelled product	
	medium content	serosal fluid content
Gen 2.5	1.9	29.8
Gen 3.5	7.2	33.1
Gen 5.5	4.8	37.9
Gen 3	8.1	29.7
Gen 4	6.7	36.6

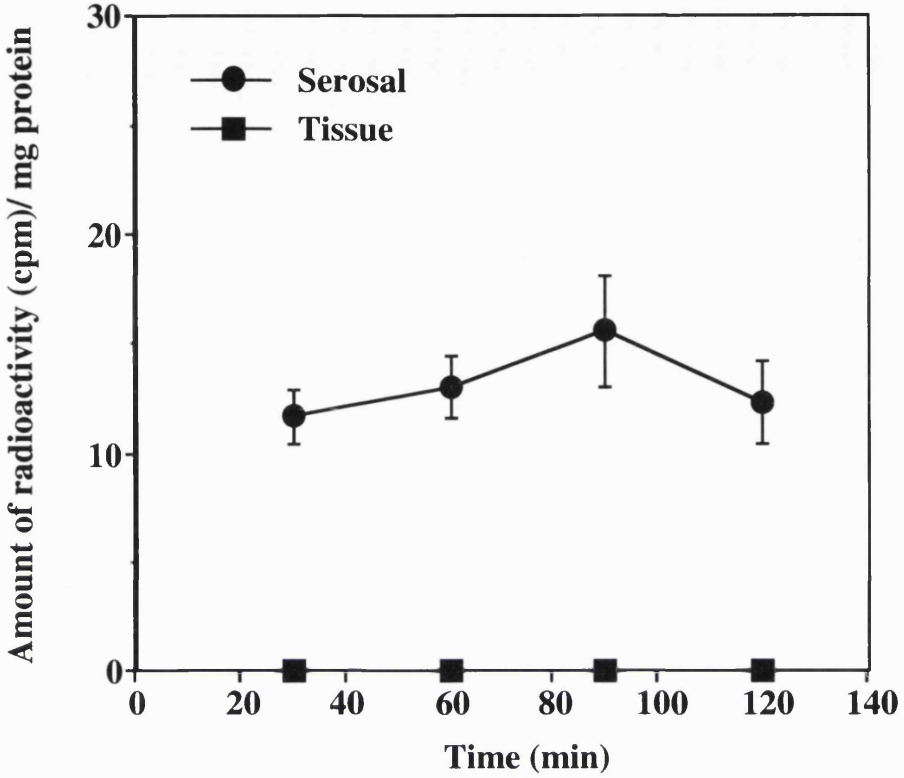


Figure 4.6. Uptake and transfer of free [^{125}I] iodide with time ; mean (n = 3) ± S.D.

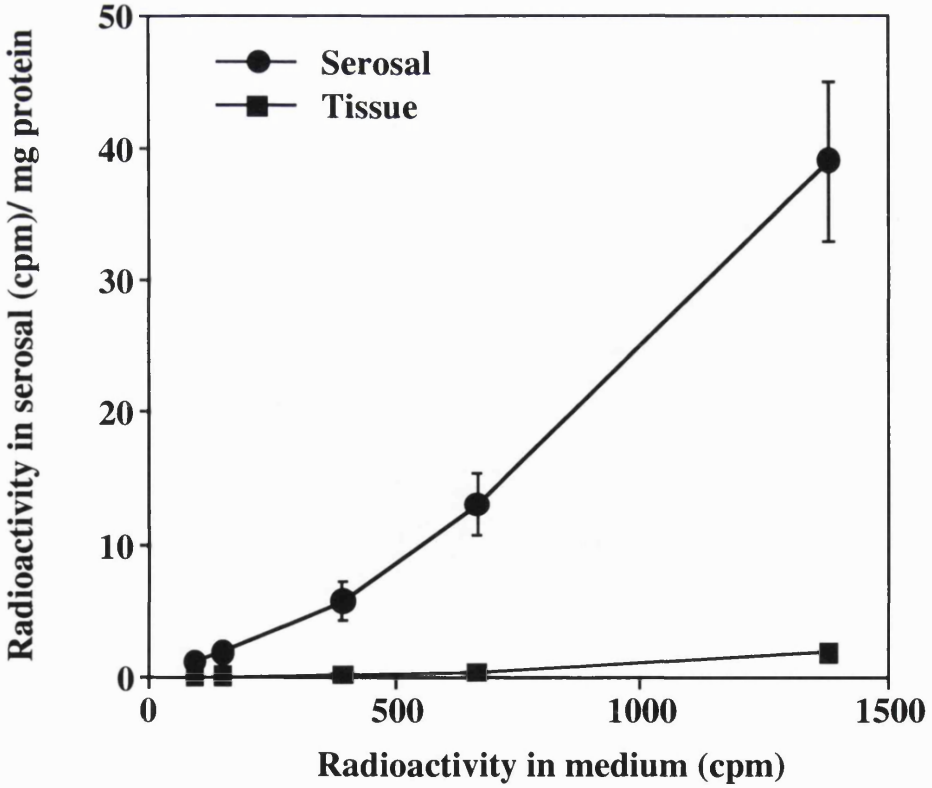


Figure 4.7. Uptake and transfer of free [^{125}I] iodide at different concentrations; mean (n = 3) ± S.D.

Table 4.6. Ability of everted adult rat intestinal sacs to concentrate glucose in the presence of PAMAM dendrimer (20 μg/ml)

Dendrimer	Ratio of glucose concentration (medium: serosal) *		
	0 min	60 min	120 min
Gen 2.5	1: 1	1: 1.22	1.87
Gen 3.5	1: 1	1: 1.98	2.43
Gen 5.5	1: 1	1: 1.46	1.89
Gen 3	1: 1	1: 1.44	1.97
Gen 4	1: 1	1: 1.32	1.75

*Each ratio represents the mean of nine values

and 0.1 - 0.3 µl/mg protein/h for serosal transfer respectively (Table 4.7). Modification of ^{125}I-labelled poly (NVPMA) to introduce cationic or anionic pendent groups elevated tissue capture approximately 6 and 4.8-fold respectively, whilst serosal transfer was elevated 16 and 20-fold respectively. The family of PAMAM dendrimers used in this study have the potential advantage that their Mw, size and the number of chain-ends have been geometrically synthesized, therefore it was possible to systematically investigate these effects on their rate of tissue association and transport across the GI mucosa.

In this study, ^{125}I-labelled PAMAM dendrimers with anionic and cationic surfaces were quite rapidly taken up by the intestinal tissue and transported across the GI epithelium. The anionic dendrimers gen 2.5 and 3.5 showed particularly rapid serosal transfer with low tissue levels indicating an efficient transport pathway (Table 4.6). The rate of uptake and transfer was constant with time and did not show saturation at over the substrate concentration used. These observations are consistent with Pato et al. (1994) who reported that anionic poly(NVPMA) copolymers were transported across tissue more rapidly than the cationic derivative and the parent polymer.

As a low Mw ^{125}I-labelled product was found in the serosal fluid contributed (30-38%), the actual quantity of transfered ^{125}I-labelled dendrimers to serosal fluid may be lower than shown in Figures 4.1-4.3. However, it should be noted that the highest proportion of radioactivity in the serosal fluid is still contributed by the radiolabelled dendrimers.

The study of the transport of free [^{125}I]iodide using the same system showed that the free [^{125}I]iodide passes across the intestinal tissue rapidly and tended to be saturated after 30-60 min incubation (Figure 4.6). As this was not the pattern seen in Figure 4.1-4.2. it is again evidence to support transfer of radiolabelled dendrimers. Although the serosal transfer increased with increasing concentration of free [^{125}I]iodide in the starting medium, the level of radioactivity recovered from tissue was always very low (0-4%) (Figure 4.7). It seems evident that the results seen in Figures 4.1-4.3 are largely due to the transfer of radiolabelled dendrimers. It is possible that the low Mw ^{125}I-labelled product that appeared in the serosal fluid came from the instability of radiolabelled dendrimer in the medium after a period of incubation and/or the degradation by enzymes in the epithelial cells. This finding suggests that another method of radiolabelling e.g. using [^{3}H] or [^{14}C] incorporated as a part of the dendrimer molecular structure may be useful to substantiate these observations.

Dendrimers, like other macromolecules, could be transported across the intestine via either the transcellular or paracellular pathways. Small hydrophilic and charged drugs

are often passively absorbed via the intercellular junctions. According to literature, the approximate size limit for this pathway in rat is ~ 1.0-1.5 nm (Nellans 1991), but others have suggested that molecules with diameter up to 3 nm may pass via this route (Pantzar et al. 1994). Therefore, it is theoretically possible that gen 2.5 and 3.5 which have small size (3.6 and 4.5 nm, respectively) and conformational flexibility might use the paracellular pathway or have the capacity to open transiently intercellular junctions. Markers such as [^{14}C]mannitol, [^{51}Cr]EDTA and radiolabelled poly(ethyleneglycol) (PEG) (400, 900 and 4000 Da) have all been routinely used as markers for paracellular transport in Caco-2 cells (Knipp et al. 1997; Yee 1997; Yu et al. 1997) and rat small intestine (Pantzar et al. 1994; Barth et al. 1998). Thus, the ability to modify the rate of transport of such markers would establish whether dendrimers have the capacity to open intercellular junctions.

It is more likely that dendrimers, like the other polymers mentioned above, are taken across enterocytes by transcytosis. The upper size limit for particle transcytosis by enterocytes reported by Jani is approximately 100 nm (Jani et al. 1992). However, the recent publications by Florence (1997) and Hussain & Florence (1998) have shown that 500 nm polystyrene particles attached with tomato lectin and invasin used this pathway for absorption. This was supported by Carreno-Gomez (1999) who suggested that 500 nm polystyrene particles coupled to tomato lectin can transverse the rat intestine by this route. Additionally, ^{125}I-labelled PVP of Mw up to ~100,000 Da has been shown to be taken up by epithelial cells by endocytosis (Duncan et al. 1980). The rate of fluid-phase endocytosis in the everted sac system used here is already well established as 0.6-1.6 µl/mg protein/h for tissue uptake. Therefore, the EI values observed for ^{125}I-labelled gen 2.5 and 3.5 (0.6-0.7 µl/mg protein/h) is consistent with uptake via this mechanism.

Generally, the mechanism of uptake of molecules can be determined by the everted sac system (Barth et al. 1999). If endocytosis is the mode of transport, the process of absorption is slow, compounds will be detected in the tissue and very small amounts in the serosal fluid. The translocation may or may not be saturable, depending on whether or not it is receptor-mediated. On the other hand, if the paracellular route is the mode of transport, there would not be any test compounds found in the tissue, they would be present at higher concentration in the serosal fluid, and the translocation should be concentration-dependent. According to the results of absorption of gen 2.5 and 3.5, the process of absorption was rapid, most compounds were detected in the serosal fluid with only a small amount in the tissue. Tissue uptake and serosal transfer increased with increasing substrate concentration. Therefore, it is possible that the combination of both pathways are used by ^{125}I-labelled gen 2.5 and 3.5. In the case of PAMAM dendrimer gen 5.5, it displayed higher tissue accumulation (2.48 ± 0.51 µl/mg protein/h) but the

similar rate of serosal transfer compared to gen 2.5 and 3.5 indicates size/conformation sensitivity of the transport mechanism. The high level detected in the tissue would suggest that these molecules attach to the invaginating plasma membrane, by specific or non-specific adsorption, and are thus taken up by adsorptive endocytosis. The levels of dendrimers detected in the serosal fluid indicated the possibility of a contribution of absorption via paracellular route, but at the low efficiency when compared with gen 2.5 and 3.5. Although gen 5.5 molecules have bigger size (6.7 nm) than those two small generations, the molecules have more globular shape and compact structure which may facilitate the transport through the tight junction.

As cationic PAMAM gen 3 and 4 have also the same size as gen 2.5 and 3.5 (3.6 and 4.5 nm, respectively), they were expected to use the same pathways. However, the uptake study showed different results indicating that the surface charge of the molecules had much influence on the absorption characteristics. The higher tissue EIs seen for the cationic PAMAM dendrimers (3.3-4.8 μl/mg protein/h) and lower serosal EIs (1.5-1.6 μl/mg protein/h) would suggest the adsorptive endocytosis. Cationic PAMAM dendrimers showed higher levels of tissue association, but they were not transported across the tissue as rapidly as the anionic dendrimers. After 60 min, the plateau of tissue and serosal levels of radioactivity associated with the cationic dendrimers indicated saturation of membrane binding sites; not surprising as the negatively charged cell membrane would interact strongly with these cationic molecules.

Bioadhesion of cationic HPMA copolymers has been reported previously in the everted sac system and also other models (McCormick et al. 1986; Bridges et al. 1988). Another explanation regarding the paracellular absorption is that the protein within the junctional complexes consists of polar amino acids with ionizable side-chains result in an electrostatic field with a negative net charge of the junctional space (Pauletti et al. 1997). Therefore, it is possible that the paracellular transport of cationic dendrimers may be retarded or blocked due to charge-charge interactions. This was supported by the work of Rubas et al. (1994). They investigated the transport properties of cyclic peptide analogues, with various net charges ranging from -3 to +1 using the Caco-2 cell culture model. They concluded that peptides with net charges of -1 and -2 are able to permeate the intestinal mucosa most efficiently.

Here the serosal transfer rates observed for the anionic PAMAM dendrimers (in the range 2.3-2.8 μl/mg protein/h) were higher than previously measured for any other macromolecules using the same experimental system (Table 4.7). The natural bioadhesive tomato lectin which has an extremely high tissue interaction (13.0 μl/mg protein/h) showed relatively low rate of serosal transfer (0.85 μl/mg protein/h) (Naisbett & Woodley

Table 4.7. Endocytic indices of different macromolecules reported in the literature compared with dendrimers

Macromolecule	Concentration (μg/ml)	Endocytic index (μl/mg protein/h)		References
		Tissue	Serosal	
Neutral Polymers				
Polyvinylpyrrolidone	2.0	0.6	0.2	Naisbett & Woodley 1994
Polyvinylpyrrolidone	2.0	0.7	0.1	Bridges et al. 1987
Poly (NVP MA)	2.0	1.6	0.1	Pato et al. 1994
HPMA copolymers	2.0	0.6	0.3	Cartlidge et al. 1987
Modified Polymers				
Poly (NVP MA) (2+)	2.0	9.6	1.6	Pato et al. 1994
Poly (NVP MA) (2-)	2.0	7.8	2.0	Pato et al. 1994
Poly (NVP MA) (C8)	2.0	16.5	2.0	Pato et al. 1994
HPMA copolymers (Mw>400K)	ns	3.2	0.5	Cartlidge et al. 1987
Proteins				
Bovine serum albumin	2.0	1.1	0.2	Naisbett & Woodley 1994
Tomato lectin	2.0	13.0	0.85	Naisbett & Woodley 1994
Dendrimers				
PAMAM Gen 2.5	20.0	0.76	4.04	This study
PAMAM Gen 3.5	20.0	0.65	3.39	This study
PAMAM Gen 5.5	20.0	2.48	4.40	This study
PAMAM Gen 3	20.0	3.33	2.34	This study
PAMAM Gen 4	20.0	3.46	2.45	This study

1994). The coating of microspheres with tomato lectin increased tissue uptake significantly (EI = 84.0 ± 6.9 µl/mg protein/h) but the serosal transfer was still low (EI = 0.7 ± 0.2 µl/mg protein/h) (Carreno-Gomez et al. 1999). Although it is certainly important to understand better the mechanism of absorption of dendrimers, the results from these *in vitro* experiments demonstrated that the PAMAM dendrimers have potential for improving oral absorption of bound/ entrapped molecules and the anionic dendrimers seem to be transported by the more efficient pathway than cationic dendrimers. It is important to confirm these observations by study their transport *in vivo* (Chapter 5).

Chapter Five

In vivo body distribution and GI transit of ^{125}I-labelled PAMAM dendrimers in rats

5.1. Introduction

In chapter 4 the rapid transfer of ^{125}I-labelled PAMAM dendrimers across intestinal tissue to the serosal fluid was observed *in vitro*. The anionic dendrimers were transported to a greater extent than the cationic dendrimers. Before moving onto incorporate specific drugs into the dendrimers, it was felt important to confirm the observation made *in vitro* and investigate ^{125}I-labelled dendrimers body distribution and also their GI transit time after oral administration.

The GI transit time and body distribution of several macromolecules proposed for use as oral drug delivery systems has already been described. For example, ^{125}I-labelled crosslinked HPMA copolymers (Cartlidge et al 1987), ^{125}I-labelled HPMA copolymer (Blundel 1995), ^{125}I-labelled tomato lectin (Naisbett & Woodley 1995), and ^{125}I-labelled NVPMA copolymer (Blundel 1995). The body distribution of the radiolabelled PAMAM dendrimers has already been investigated after i.v. or i.p. administration. For example Malik et al (1999) described the body distribution of ^{125}I-labelled anionic PAMAM dendrimers gen 2.5, gen 3.5 and gen 5.5 and ^{125}I-labelled cationic PAMAM dendrimers gen 3 and gen 4, and Roberts et al (1996) described the body distribution of [^{14}C]anionic PAMAM dendrimers gen 3, gen 5 and gen 7. As described in Chapter 1 (section 1.5.4), they found that the anionic ^{125}I-labelled PAMAM dendrimers displayed longer circulation times than cationic PAMAM dendrimers (15-40 % of the recovered dose in blood at 1 h) and showed significant liver accumulation. PAMAM dendrimer gen 7 had an extremely high urinary excretion whereas the smaller dendrimer accumulated in liver, kidney and spleen.

This is the first study of PAMAM dendrimer fate after oral administration. Here the GI transit and body distribution of ^{125}I-labelled PAMAM dendrimers were followed after oral administration to adult rats. PAMAM dendrimers gen 2.5 and 3.5 were chosen as they displayed high uptake into the serosal fluid *in vitro* (Chapter 4) and PAMAM gen 3 was also used to study in order to allow comparison of the fate of anionic and cationic molecules. The stability of the radiolabelled PAMAM dendrimers in simulated gastrointestinal fluid was also investigated. When using ^{125}I-labelled probes it is always important to ensure that the preparations used do not contain free [^{125}I]iodide or low Mw degradation products and also confirm the macromolecular native of the radioactivity after administration *in vivo*.

5.2. Methods

^{125}I-Labelled anionic PAMAM dendrimers gen 2.5, gen 3.5 and ^{125}I-labelled cationic PAMAM dendrimer gen 3 (1.25 mg in 0.5 ml or 5 mg/kg) were administered by oral gavage to adult male Wistar rats (n = 3). After 1, 5 and 24 h the animals were

killed and organs (Table 5.1) removed and prepared for radioactivity measurements by γ-counter, as described in section 2.2.9. Urine and faeces samples (24 h time point) were analysed by sephadex G25 column chromatography to examine the nature of the radioactivity in the sample as detailed in section 2.2.9.

The stability of ^{125}I-labelled dendrimers in simulated gastrointestinal fluid was studied by incubating the radiolabelled dendrimers in simulated gastric fluid pH 1.2 (USP) and in simulated intestinal fluid pH 7.5 (USP) as described in section 2.2.9. The samples were then analysed using the sephadex G25 column chromatography and the elution profile obtained compared with that of the starting preparation of ^{125}I-labelled dendrimers. The percentage of low Mw radioactivity was then calculated.

Statistical differences were estimated using the Student's t-test.

5.3. Results

5.3.1. Body distribution and GI transit

PAMAM gen 2.5

The tissue distribution of radioactivity recovered following oral administration of ^{125}I-labelled PAMAM gen 2.5 is shown in Table 5.2 and Figure 5.1. After 1 h, almost all the radioactivity recovered was located in the stomach and small intestine. The majority of the activity was recovered from gut washing (~ 90%) rather than gut tissue (~ 10%). After 5 h, there was almost no radioactivity left in the stomach and small intestinal-associated radioactivity had fallen to less than 10%. Most of the radioactivity was transfered to caecum and colon with approximately 50% in the colon-washings. After 24 h, 80-90% of the dose was found in faeces and ~10% was retained in the large intestine. The small amount of activity was found in urine. The total radioactivity recovered at each time point was approximately 85-90% of administered dose.

PAMAM gen 3.5

Similar results to those seen for PAMAM gen 2.5 were obtained for PAMAM gen 3.5 as shown in Table 5.3 and Figure 5.2. One hour after oral dosing, most of the radioactivity was recovered in the stomach and small intestine with the majority recovered from the washings. After 5 h, about 70 - 80% of the radioactivity had moved to the caecum and colon, with 23.2 ± 9.4% remaining in small intestine. The amount of radioactivity left in small intestine after 5 h (23.2 ± 9.4%) was higher than that seen for gen 2.5 (6.8 ± 1.8%) ($p < 0.05$). By 24 h, approximately 70 - 80% of the dose was found in faeces and there was no significant difference in the amount of radioactivity

Table 5.1. Organs removed for the radioactivity measurement

Organ	Abbreviation
Blood	Bl
Liver	Li
Lungs	Lu
Kidneys	K
Heart	H
Spleen	Sp
Stomach + washing	St + StW
Small intestine + washing	Sm + SmW
Caecum	C
Colon + washing	Co + CoW
Urine	U
Faeces	F

Table 5.2. Body distribution of ^{125}I-labelled PAMAM gen 2.5 after oral administration to rats

Organ	Time		
	1 h	5 h	24 h
Blood	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
Liver	0.0	0.1 ± 0.1	0.1 ± 0.2
Lungs	0.0	0.0	0.0
Kidneys	0.0	0.0	0.0
Heart	0.0	0.0	0.0
Spleen	0.0	0.0	0.0
Stomach	3.5 ± 2.8	0.1 ± 0.0	0.0
Stomach washings	37.5 ± 9.2	0.6 ± 0.1	0.2 ± 0.2
Small intestine	5.3 ± 2.3	0.3 ± 0.1	0.0
S.I.washings	53.3 ± 9.8	6.8 ± 1.8	0.8 ± 0.3
Caecum	0.0	45.4 ± 14.1	4.9 ± 4.3
Colon	0.0	1.7 ± 0.1	1.1 ± 1.7
Colon washings	0.1 ± 0.1	44.8 ±15.6	3.9 ± 3.7
Urine	0.0	0.1 ± 0.1	1.4 ± 0.9
Faeces	0.0	0.0	87.1 ± 6.7
Recovery of dose administered (%)	90.3 ± 7.3	91.4 ± 9.2	86.4 ±17.0

Results are expressed as % of the total dose recovered; mean ± S.D. (n = 3)

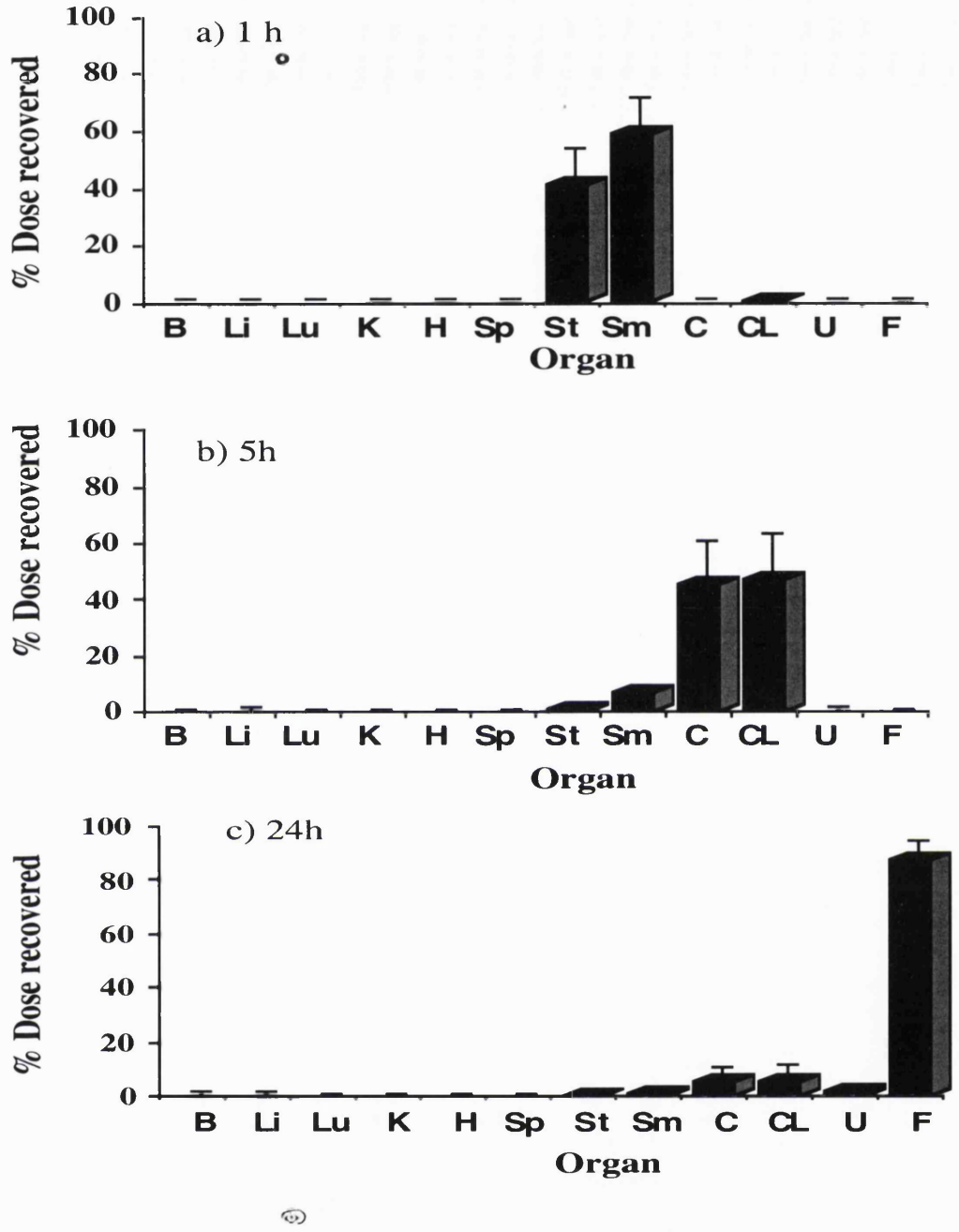


Figure 5.1. Body distribution and GI transit of ^{125}I-labelled PAMAM gen 2.5 . Data represent the mean (n = 3) ± S.D. The values present for GI organs are the sum of the values in the tissue and the lumen washings

Table 5.3. Body distribution of ^{125}I-labelled PAMAM gen 3.5 after oral administration to rats

Organ	Time		
	1 h	5 h	24 h
Blood	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.1
Liver	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
Lungs	0.0	0.0	0.0
Kidneys	0.0	0.0	0.0
Heart	0.0	0.0	0.0
Spleen	0.0	0.0	0.0
Stomach	1.4 ± 0.9	0.2 ± 0.2	0.1 ± 0.1
Stomach washings	35.7 ± 9.8	1.7 ± 1.3	0.5 ± 0.6
Small intestine	2.8 ± 1.6	0.6 ± 0.4	0.1 ± 0.1
S.I.washings	59.6 ± 8.5	23.2 ± 9.4	4.9 ± 4.3
Caecum	0.0	50.2 ± 2.2	7.3 ± 9.2
Colon	0.0	0.9 ± 0.6	2.1 ± 1.6
Colon washings	0.1± 0.2	22.2 ± 11.6	5.6 ± 3.7
Urine	0.0	0.4 ± 0.2	1.6 ± 0.8
Faeces	0.0	0.0	77.5 ± 14.0
Recovery of dose administered (%)	72.4 ± 10.0	62.8 ± 5.9	69.4 ± 2.3

Results are expressed as % of the total dose recovered; mean ± S.D. (n = 3)

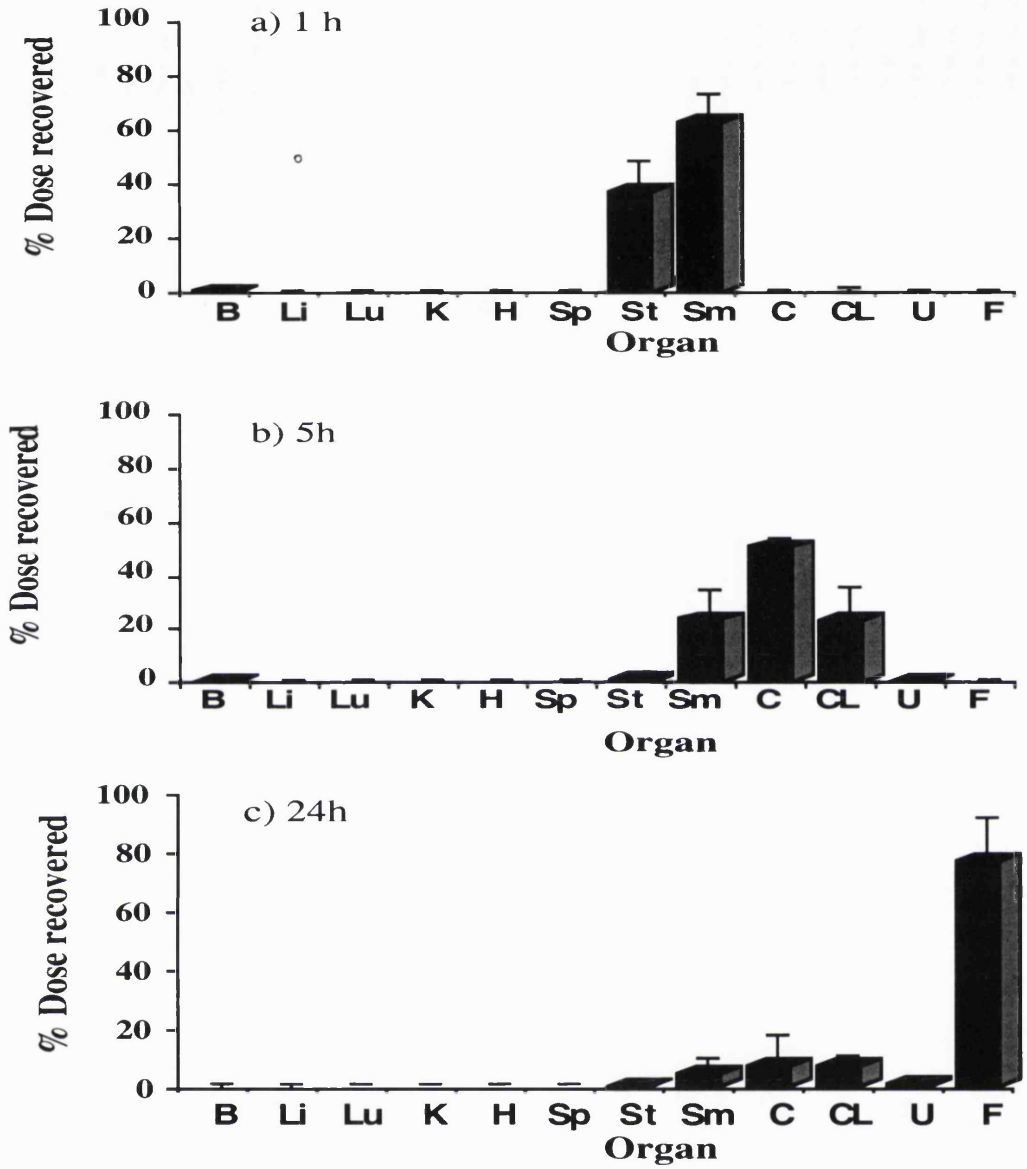


Figure 5.2. Body distribution and GI transit of ^{125}I-labelled PAMAM gen 3.5 . Data represent the mean (n = 3) ± S.D. The values present for GI organs are the sum of the values in the tissue and the lumen washings

detected in faeces between gen 2.5 and gen 3.5 ($p < 0.05$). The small amount of activity was also found in urine. The total radioactivity recovered at each time point was approximately 60-70% of administered dose.

PAMAM gen 3

The GI transit and body distribution of ^{125}I-labelled PAMAM gen 3 are shown in Table 5.4 and Figure 5.3. The behaviour and pattern of distribution were similar to those of anionic dendrimers. One hour after administration, most of radioactivity was located in the stomach and small intestine with very small amount in the blood (~1%). Transfer into caecum and colon was seen after 5 h, with 7.7 ± 4.6% remaining in the small intestine which was not different from that seen for gen 2.5 (6.8 ± 1.8%) ($p < 0.05$) and there was a small amount of radioactivity found in urine (2.5 ± 0.8%). Most of the radioactivity was recovered in the faeces (~70%) and the small amount found in the urine (7.8%) after 24 h. The total radioactivity recovered at each time point was approximately 65-80% of administered dose.

5.3.2. GPC of urine and faeces samples

The elution profiles of the urine and faeces samples after 24 h are presented in Figures 5.4 - 5.6. The profiles obtained for gen 2.5 and gen 3.5 showed that most of the radioactivity in faeces could be attributed to ^{125}I-labelled dendrimer and a relatively small amount represented low Mw ^{125}I-labelled products were presented. In the case of gen 3 the amount of the low Mw ^{125}I-labelled product detected in faeces was approximately half of the recovered radioactivity. The characterisation of the radioactivity in urine showed the present of low Mw molecule. For PAMAM gen 2.5, there was a small amount of ^{125}I-labelled dendrimer found also in urine (Figure 5.4c).

5.3.3. Stability of radiolabelled dendrimers in the GI tract

Incubation of radiolabelled dendrimer with simulated gastric fluid 1 h or intestinal fluid 5 h resulted in the liberation of low Mw ^{125}I-labelled products (~ 5-7% in gastric fluid and ~ 4-9% in intestinal fluid (after subtraction by the amount presented in the samples before incubation) (Table 5.5).

5.4. Discussion

Oral administration of ^{125}I-labelled PAMAM dendrimers clearly showed the transfer of radioactivity along the GI tract, generally most of the dose recovered was found in the stomach and small intestine in 1 h and it was transferred to the caecum and colon after 5 h. By 24 h most of the radioactivity was located in faeces. ^{125}I-Labelled PAMAM dendrimers displayed a much slower transit time than was observed for neutral, linear polymers such as HPMA copolymer and NVPMA copolymer (Blundell

Table 5.4. Body distribution of ^{125}I-labelled PAMAM gen3 after oral administration to rats

Organ	Time		
	1 h	5 h	24 h
Blood	1.2 ± 0.5	1.0 ± 0.1	0.3 ± 0.2
Liver	0.5 ± 0.1	0.3 ± 0.0	0.1 ± 0.1
Lungs	0.1 ± 0.0	0.1 ± 0.1	0.0
Kidneys	0.1 ± 0.0	0.1 ± 0.0	0.0
Heart	0.0	0.0	0.0
Spleen	0.0	0.0	0.0
Stomach	1.6 ± 0.8	0.3 ± 0.1	0.3 ± 0.1
Stomach washings	27.7 ± 20.6	1.3 ± 0.9	2.5 ± 2.2
Small intestine	1.9 ± 1.5	0.4 ± 0.2	0.1 ± 0.1
S.I.washings	66.7 ± 21.8	7.7 ± 4.6	1.9 ± 1.8
Caecum	0.1 ± 0.1	33.5 ± 5.5	8.0 ± 7.1
Colon	0.1 ± 0.1	1.7 ± 1.0	0.2 ± 0.2
Colon washings	0.1 ± 0.1	51.6 ± 7.7	7.2 ± 7.3
Urine	0.0	2.5 ± 0.8	7.8 ± 2.9
Faeces	0.0	0.0	70.2 ± 8.0
Recovery of dose administered (%)	68.1 ± 3.6	65.4 ± 13.6	72.0 ± 14.5

Results are expressed as % of the total dose recovered; mean ± S.D. (n = 3)

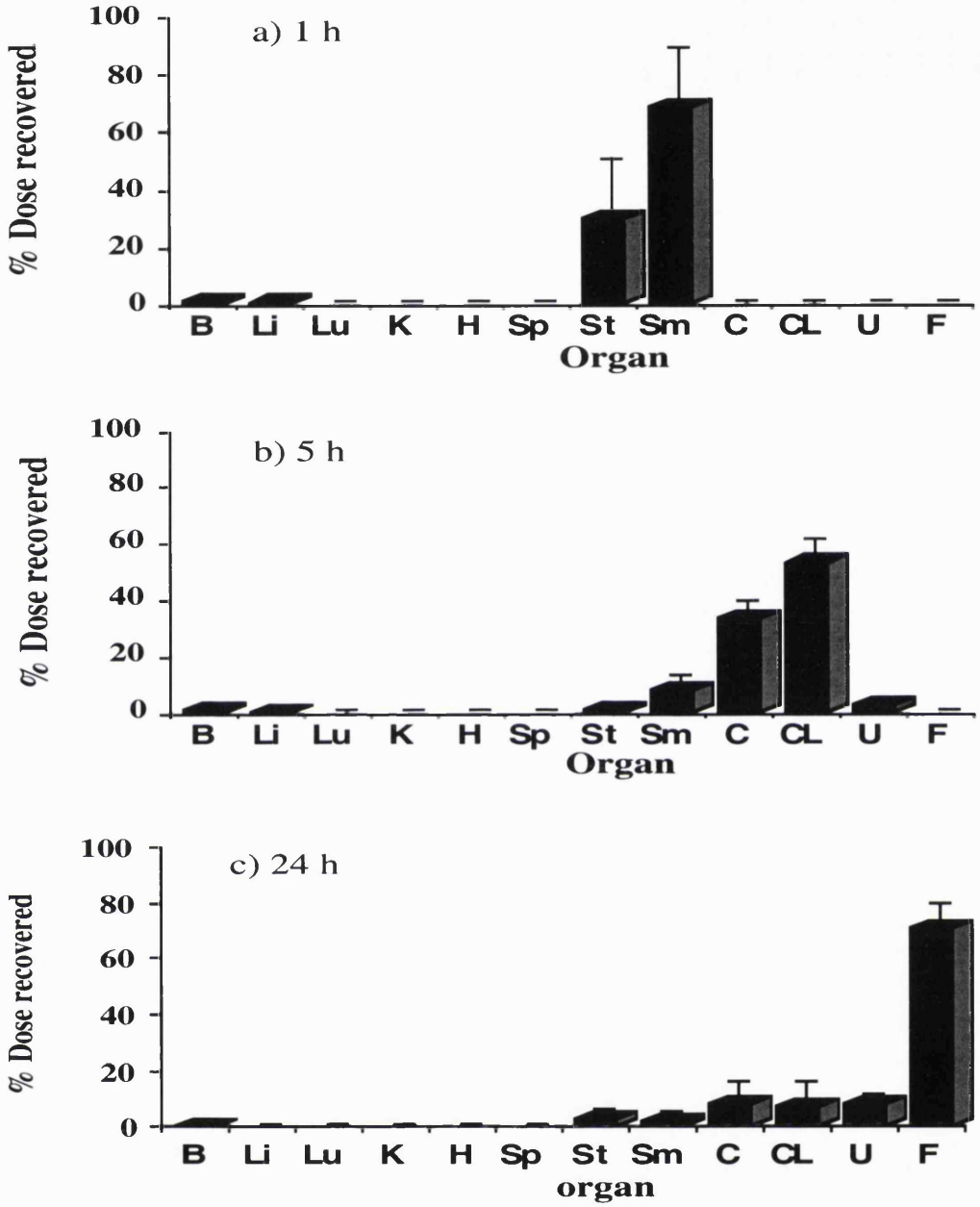


Figure 5.3. Body distribution and GI transit of ^{125}I-labelled PAMAM gen 3. Data represent the mean (n = 3) ± S.D. The values present for GI organs are the sum of the values in the tissue and the lumen washings

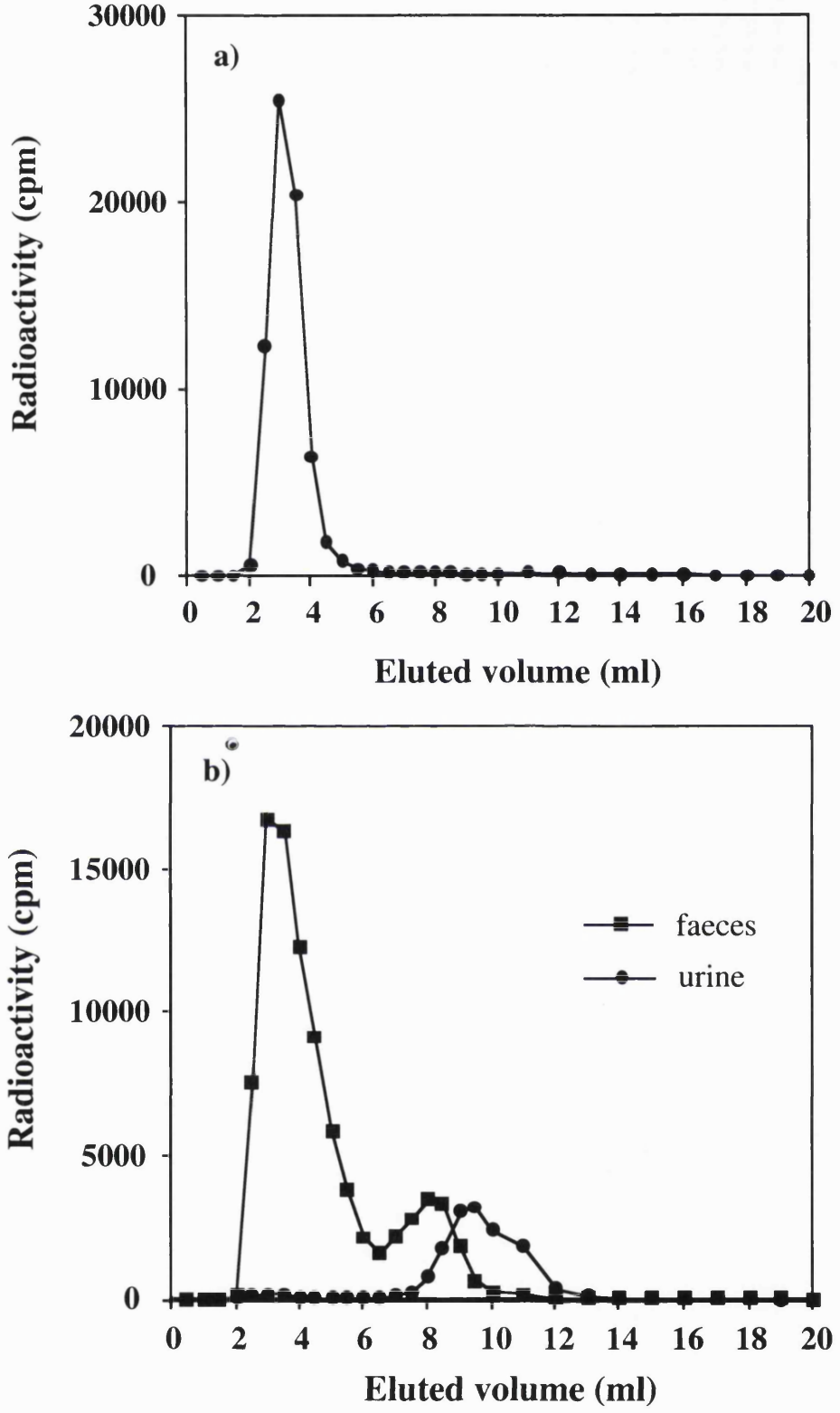


Figure 5.4. Elution profiles of (a) ^{125}I-labelled PAMAM gen 2.5, (b) faeces and urine samples

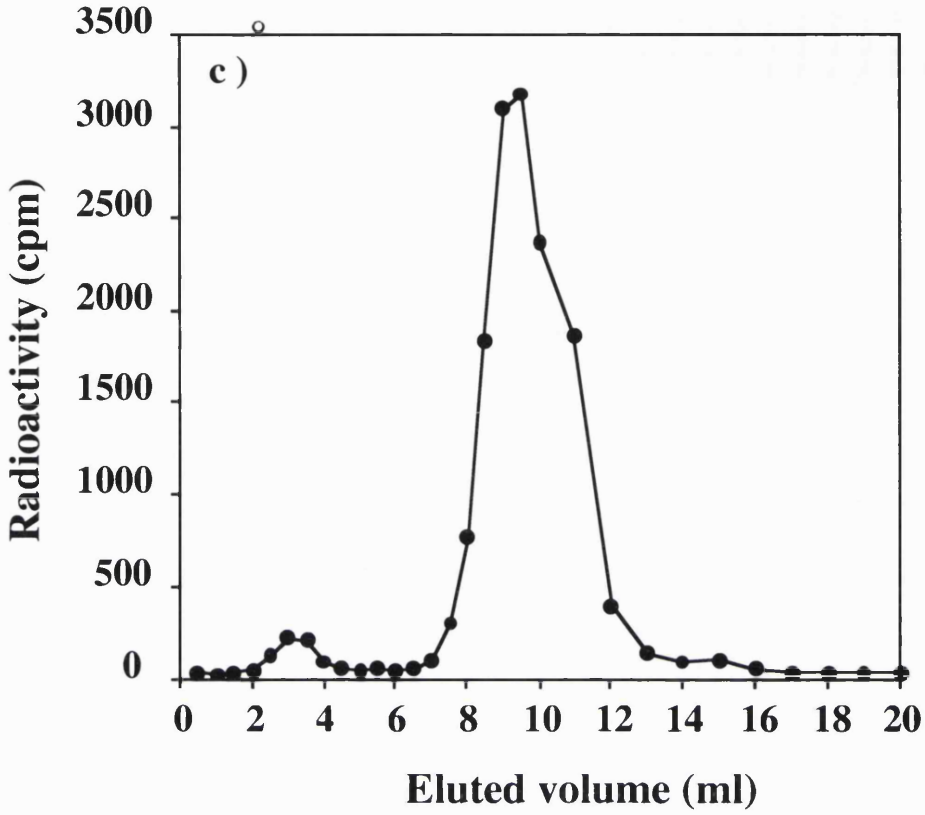


Figure 5.4. (continued) Elution profile of (c) urine sample (enlarged)

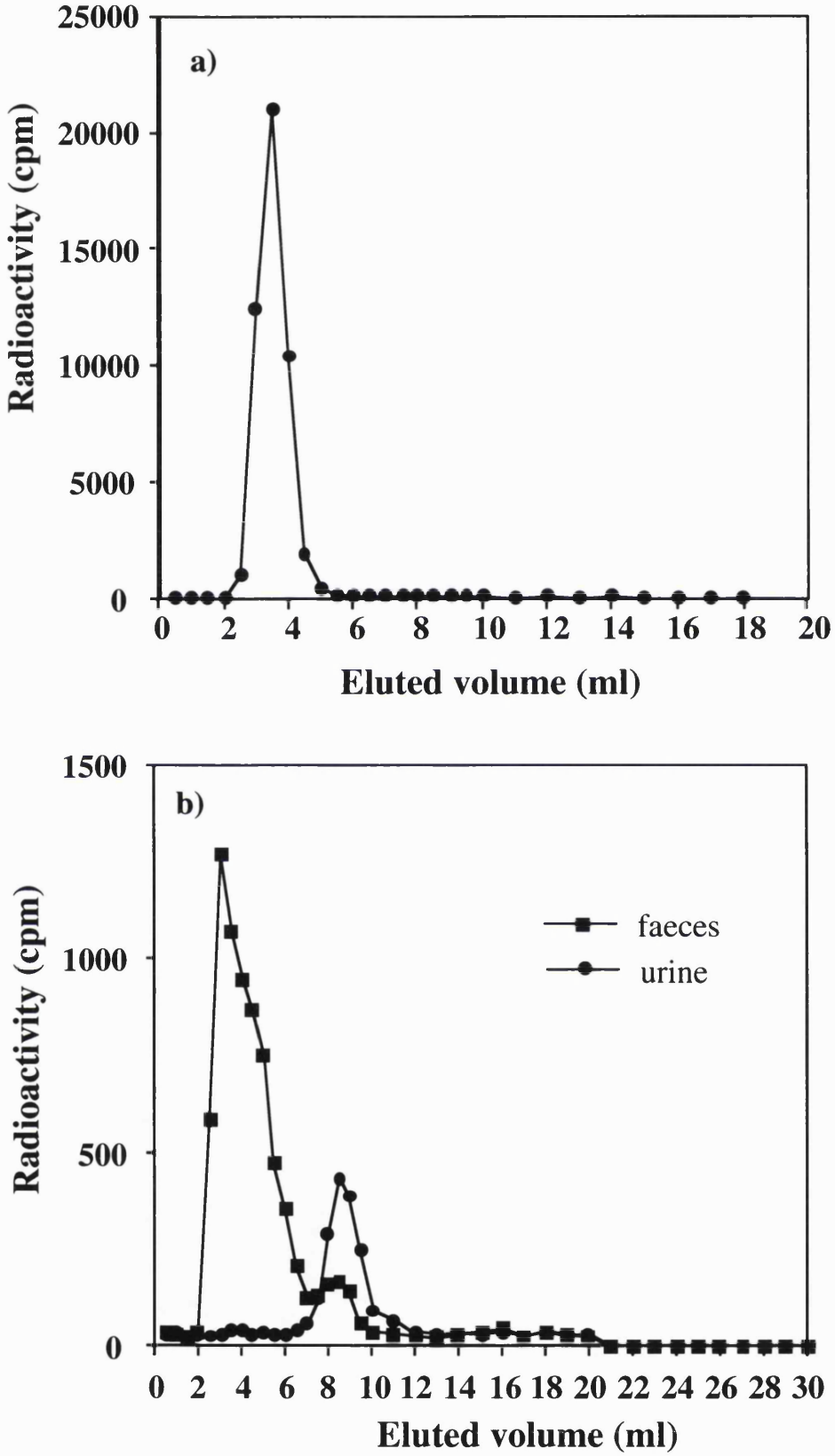


Figure 5.5. Elution profiles of (a) ^{125}I-labelled PAMAM gen 3.5, (b) faeces and urine samples

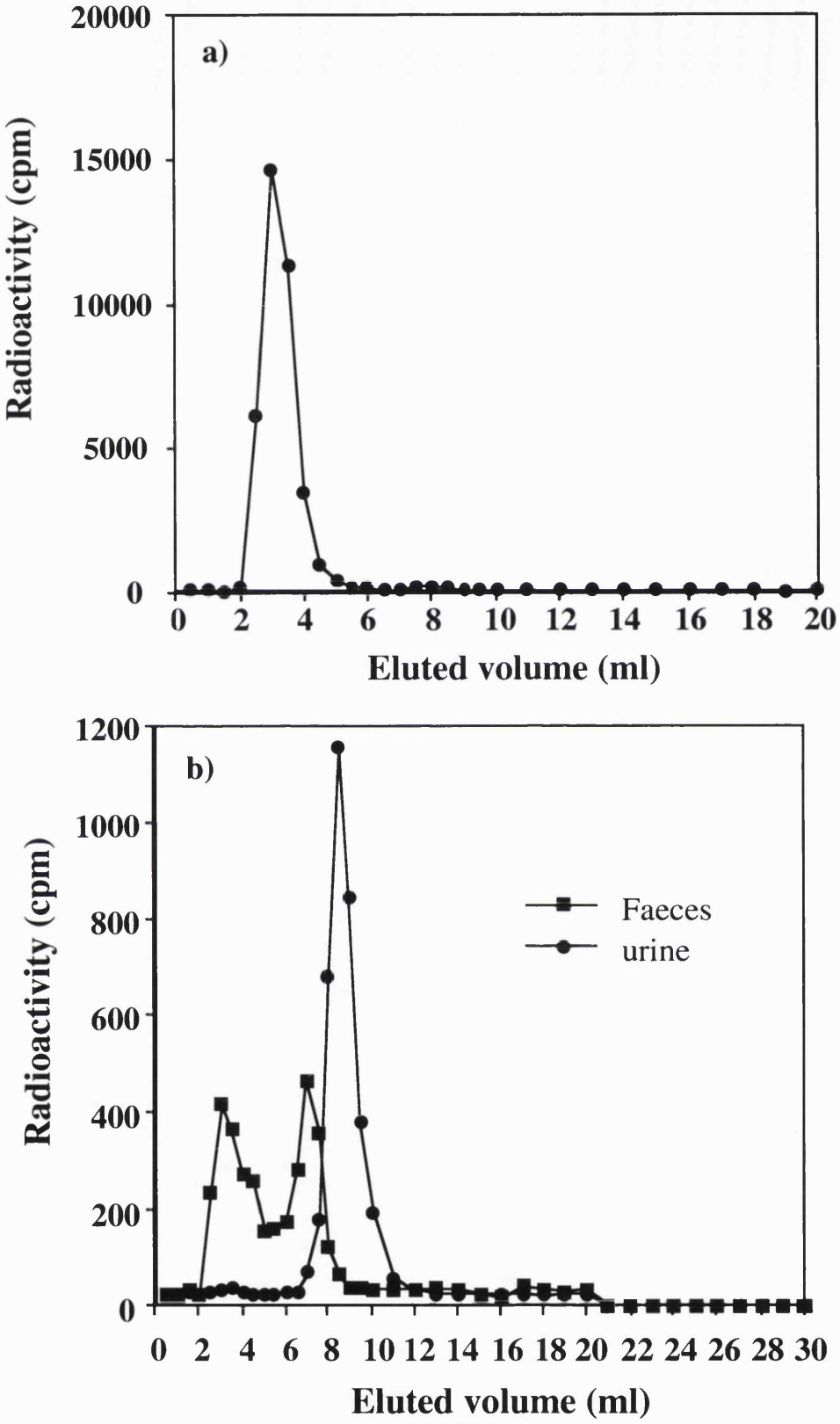


Figure 5.6. Elution profiles of (a) ^{125}I-labelled PAMAM gen 3, (b) faeces and urine samples

Table 5.5. Stability of radiolabelled dendrimer after incubation in simulated GI fluids by GPC

Dendrimers	% radioactivity in low Mw peak		
	Before incubation	After incubation in gastric fluid (1 h)	After incubation in intestinal fluid (5 h)
gen 2.5	1.1	8.0	8.3
gen 3.5	1.7	9.5	6.2
gen 3	2.0	7.2	11.1

1995). These polymers displayed a rapid transit; most of the dose was already localised in the distal small intestine after 1 h (StW = 10.1% for HPMA copolymer and 13.4% for NVPMA). In the case of ^{125}I-labelled PAMAM dendrimers gen 2.5, gen 3.5 and gen 3; about 30-40% of the radioactivity was still left in stomach after 1h. This observation confirms previous studies where it was shown that incorporation of charged residues into a polymer can result in a delayed GIT transit time (Blundell 1995). However, generally there was no marked difference in the transit time of cationic and anionic dendrimers gen 2.5 and gen 3, although the bigger anionic dendrimers PAMAM gen 3.5 showed longer intestinal transit time than the other two gens. The amount of radioactivity left in the small intestine after 5 h for gen 3.5 (23.2 ± 9.4%) was higher than that seen for gen 2.5 (6.8 ± 1.8%) and gen 3 (7.7 ± 4.6%).

The characterisation of the radioactivity recovered from faeces indicated that ^{125}I-labelled gen 2.5 and gen 3.5 were resistant to degradation during passage along GI tract. However, it appeared that approximately 50% of gen 3 was degraded to smaller fragments. The rather broad peaks of the elution profiles seen on sephadex G-25 are probably due to the many compounds contained in faeces. This made it difficult to identify whether these degradation products were [^{125}I]iodide or radiolabelled degradation fragments of dendrimers themselves. There is also a possibility that the radioactivity could adsorb onto compounds within faeces thus altering the elution profile. In the case of ^{125}I-labelled PAMAM gen 2.5, a small amount of radiolabelled dendrimer was also found in urine. All dendrimers in this experiment have Mw in the range of 6,011-12,419 Da and this is small enough to allow the molecule pass through the kidney by glomerular filtration. The Mw cut-off for the kidney is approximately 40-50 kDa depending on molecular size, charge and shape (Smith et al. 1983). Therefore if dendrimers are transferred across the GI mucosa into the circulation and they are non-degradable, we would expect to find some ^{125}I-labelled dendrimer in urine. However, it should be noted that the total radioactivity counts detected in the urine was rather low.

Evaluation of the body distribution of ^{125}I-labelled PAMAM dendrimers after oral administration showed limited direct evidence of dendrimer absorption across the GI tract, only a small amount of gen 2.5 was found in urine. However, the total recovery of radioactivity administered was always in the range 70-90%. Therefore, it is not yet clear what is the fate of the "missing" fraction of radiolabelled dendrimers.

Compared with the results obtained from the *in vitro* experiments using the everted intestinal sac method, the uptake *in vivo* seemed to be much lower. For example, *in vitro* the amount of gen 3.5 transferred to serosal at 60 min was 68 ng/mg protein (Chapter 4). If we try to calculate the total absorption for the whole intestine, the

wet weight of intestinal tissue is approximately 8.0 g. This is approximately equivalent to 772.9 mg protein. Thus, the theoretical transfer of dendrimers when compared to that seen *in vitro* would be 52.55 μg or 26.3% of the given dose. (It is interesting that this value is similar to the amount of radioactivity labelled dendrimer not yet accounted for in their studies). This calculation assumes that the concentration of substance is the same throughout the whole intestine, and that the transport rate is constant for every part of intestine which are unlikely to be the case. Also it should be noted that the everted sac is a closed system in which the mucosal surface is exposed directly to the substrate. During preparation, the mucosal surface area of the sac may be extended by the medium inside and therefore a large tissue surface area is in contact with the substrate over the incubation time. Moreover, the macromolecule which is transfered into serosal compartment will stay inside the sac accumulating there. Thus, it makes it possible to measure the actual amount of transfer substance. In contrast, the exposed surface area and contact time of intestinal tissue with ^{125}I-labelled dendrimers in an *in vivo* experiment will be much less. The dendrimers is moving along the GI tract due to peristalsis. Also the thick layer of mucous presents a physical barrier sometimes up to 100 μm deep. The dendrimer would have to diffuse through this barrier and may stick to it. *In vivo*, if a macromolecule is transferred across the gut, it will be passed to the systemic circulation which is an open system and contains a large volume for distribution, so a small amount of material absorbed would be hardly detectable.

It has been reported that many soluble polymers for example HPMA copolymers, and NVPMA copolymers are not transported across the intestine into the blood circulation in significant quantities (Cartridge 1987; Blundell 1995). Although the NVPMA copolymers displayed higher tissue uptake and serosal transfer than HPMA copolymers in *in vitro* rat everted intestine model, they performed no better *in vivo* than the HPMA copolymers. In everted sac system, tomato lectin displayed high tissue uptake and low serosal transfer (Naisbett & Woodley 1994) (Table 4.7) but seemed to be transferred into systemic circulation at higher rates than seen for other macromolecules including the PAMAM dendrimers in this study. (There was only 50 % lectin in the GI tract after 5 h and the large amount of radioactivity detected in urine (Naisbett & Woodley 1995)). However, the nature of the radioactivity detected in urine suggested a large extent of lectin degradation in the GI tract. Little difference in the transit time was seen for lectin and control molecule (PVP) though the *in vitro* data showed that tomato lectin could bind avidly to small intestinal gut rings which may prolong GI transit. This was explained by the possibility of the interaction of tomato lectin with intestinal mucus thus preventing interaction with the enterocyte surface.

In the case of nanoparticles, the amount of systemic absorbtion after single dose administered was rather low. For example, the transfer of polymethylmethacrylate nanoparticles (diameter 130 ± 30 nm) was 1-3 % of the administered dose (Araujo et al. 1999). However, the work by Jani et al. (1990) showed that repeating dose of PLGA nanoparticles (diameter 50 nm) for 10 days resulted in the systemic uptake of 7 %.

The liberation of the small amount of low Mw [^{125}I]-labelled product after incubation in simulated GI fluid demonstrated the potential instability of the radiolabel in GI fluid. As the GPC of urine taken from animal given ^{125}I-labelled PAMAM dendrimer gen 3 and gen 3.5 showed only low Mw [^{125}I] species. It is possible that some radiolabel [^{125}I]iodine became detached in the GI tract or that the radiolabelled dendrimers themselves were degraded into small fragments possibly by pH, bacteria, or enzymes. Further studies are required to explain these observations.

The results from *in vivo* body distribution of PAMAM dendrimers after a single dose oral administration showed a low level of absorption compared to the results from the *in vitro* experiments (Chapter 4). However, GPC of urine samples suggest that small amounts of gen 2.5 might be absorbed and excreted. In addition, approximately 10-30% of the administered dose was not found in any of the organs studied. Therefore, the *in vivo* results must so far be considered inconclusive. In future experiments it is proposed to use gamma scintigraphy (reviewed by Pimm 1999) to give both qualitative and quantitative measurement of the fate of radiolabeled dendrimers after oral administration. Although a PAMAM dendrimer gen 3.5 containing DTPA has already been prepared time did not permit the gamma camera studies, but this will be completed later. Another way to investigate whether PAMAM dendrimers can promote oral absorption is to study the fate of dendrimer-drug complexes. Therefore it was decided to prepare and characterise such complexes to enable further studies (Chapter 6).

Chapter Six

Complexation of piroxicam and indomethacin with PAMAM dendrimers

6.1. Introduction

Dendrimers have been proposed as nanoparticulate drug carriers and can theoretically carry drugs either by binding them to the surface of the molecule or by entrapping them within the internal cavity (Tomalia et al 1985). Therefore, they may be useful for different kinds of application in the field of drug delivery, including enhancing the solubility of poorly soluble drugs and also controlled drug release (Tomalia 1995). The possibility of constructing a dendritic core-shell molecule to entrap small molecules was first proposed by Maciejewski (1982). This has been discussed as one of the main theoretical applications of dendrimers ever since, but there are still no practical studies demonstrating that this concept can really be useful. Complexation of drugs with dendrimers can be classified as two distinct approaches; complexation of drug in the dendrimer 'interior' or complexation of drug at the dendrimer 'surface'. Drug complexation within the core can be facilitated by one or a combination of the following mechanisms:- 1) hydrophobic interaction, 2) hydrogen bonding, 3) physical encapsulation, and 4) metal ion coordination (reviewed by Zeng & Zimmerman 1997). The first three mechanisms can be particulary important in the devolopment of a drug delivery system and will be briefly reviewed here.

Interaction of drugs and model compounds with dendrimers

Hydrophobic interactions

When the surface of an apolar dendrimer contains charged functional groups, its gross structure resembles that of a micelle which allows the encapsulation of a lipophilic compound. The essential criterion is simply that the enclosed molecule or "guest" be a suitable size and shape to fit into a cavity within a solid structure formed by the "host" molecule. The stereochemistry, and possibly, the polarity of both the host and the guest molecules determine whether inclusion can occur (Esfand et al 1996). Frechet (1994) reported a unimolecular micelle based on his polyarylether dendrimers. The 32 carboxylate groups on the dendrimer surface made it soluble in water and two molecules of dendrimer was able to solubilize 1 molecule of an apolar organic molecule pyrene in water. Stevelmans and co-workers (1996) reported the synthesis of inverted unimolecular dendritic micelles. They reacted the terminal amine groups of DAB dendrimers with aliphatic chains, resulting in a hydrophobic outer shell with a hydrophilic interior. This dendrimer was able to solubilize hydrophilic molecules such as Bengal Rose when they were mixed together in a hexane solution. Tomalia's group (Naylor et al. 1989) used NMR relaxation measurements to study the interaction of aspirin and 2,4-dichlorophenoxyacetic acid with carbomethoxy-terminated PAMAM dendrimer gen 0.5 - 5.5. The authors suggested interaction with the interior of the dendrimer, though they noted that the guest molecules may not be fully encapsulated but rather might congregate at the dendrimer surface (Naylor et al. 1989).

Hydrogen bonding

Hydrogen-bonding is a common phenomenon in the biological systems. Whereas the hydrogen-bonding that allows recognition of synthetic receptors in water by small molecules is remarkably inefficient (Smith & Diederich 1998), it might be expected that the reduced polarity inside water-soluble dendrimers would strengthen hydrogen bonding enough to allow complexation to occur. Recently, Newkome and coworkers (1996) have synthesized a series of dendritic hosts with diacyl-amide pyridine hydrogen-bonding units. These hydrogen-bonding units contain a donor-acceptor-donor hydrogen-bonding motif which was shown to be bind to the two acceptor-donor-acceptor sites on barbituric acid. Host-guest inteactions in this system were evaluated by ^{1}H NMR titration. In the case of the higher gen dendrimer hosts, the measurement of the interaction was complicated by self-association of the dendrimers themselves molecules and also binding of the guest molecules at other sites (not hydrogen bonding) within the host.

Physical encapsulation

The physical encapsulation of guest molecules within the dendrimer interior was first demonstrated by Jansen et al (1994). They called their system "dendritic boxes". The closed dendrimer surface was constructed via the reaction of a DAB dendrimer with 64 amine surface groups with various activated Boc- or Fmoc-protected amino acids. Guest molecules, such as 7,7,8,8-tetracyano-p-quinodimethane (TCNQ) or the dye Bengal Rose were entrapped during the synthetic reaction. Once the dendrimer box was prepared the diffusion of the guest molecules out was immeasureably slow. The size/structure of the guest used and the architecture of the dendrimer cavity determined the number of guest molecules that could become entrapped within the dendritic box. It was also demonstrated that a shape-selective release of entrapped guest molecules could be achieved by removing the shell in two steps (Jansen et al 1995a). First, by partially perforating the shell using formic acid and subsequent dialysis, the smaller guest molecules were liberated whilst the larger guest was still entrapped. Second, after complete removal of the shell by 2N HCl and subsequent dialysis, the larger guest molecules were liberated. Proposed uses of the "dendritic box" include drug delivery systems for transport and controlled-release of drugs. However, this concept has never been tested practically.

Complexation at the dendrimer " surfaces "

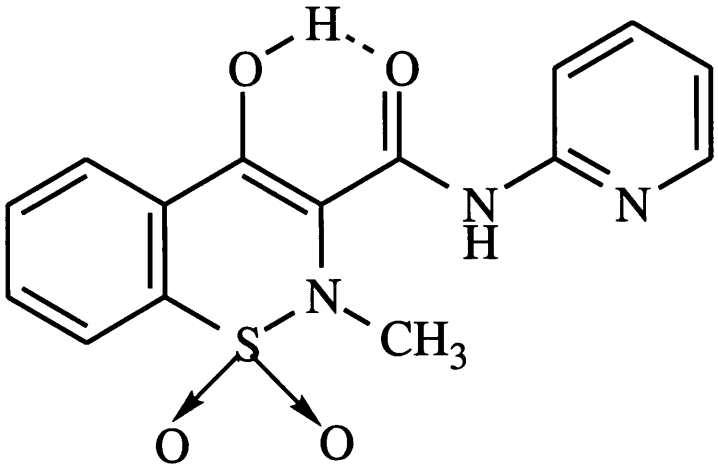
Dendrimers contain a large number of identical end groups at their surface which also provide potential sites for the complexation of drug. Complexation might theoretically occur by electrostatic interaction. When the dendrimer terminal groups are charged, the surface can behave as a polyelectrolyte and is likely to interact electrostatically with oppositely charged molecules. It has been noted that a sufficiently

large dendrimer surface with high charge density is required for strong complex formation (reviewed by Zeng & Zimmerman 1997). The possibility of complexing a linear polycations, such as poly (dimethyldiallylammonoium chloride), with carboxylate terminated PAMAM dendrimer gen 7.5 has been reported (Li & Dubin 1995). Also the electrostatic interaction of the cationic amine-terminated PAMAM dendrimers with polyanionic DNA has been demonstrated and these dendrimer-DNA complexes have been used to promote gene delivery (Haensler & Szoka 1993; Kukowska-Latallo et al. 1996).

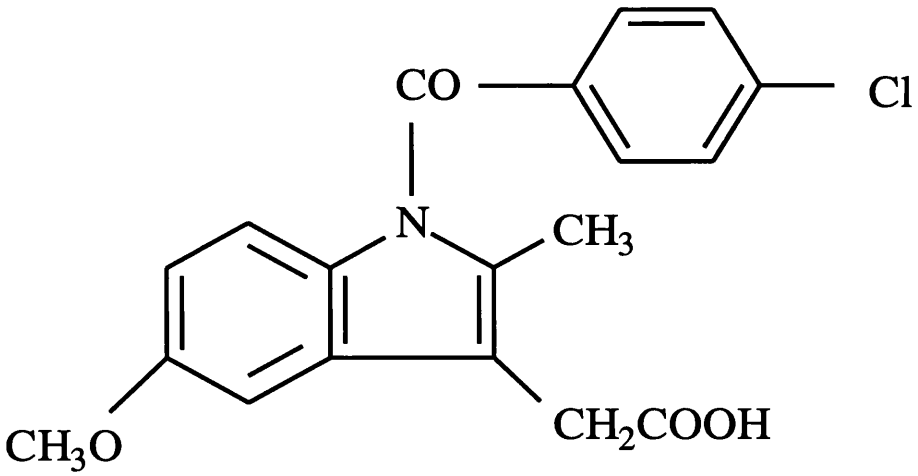
Non-steroidal anti-inflammatory drugs (NSAIDS) are important first-line drugs in the palliative treatment of acute and chronic inflammation. However, the majority of the drugs in this class are poorly soluble in water resulting in a reduction in the bioavailability of active material after oral administration. Furthermore, it is known that irritation in the GI tract occurs due to long-term exposure to high concentration of drugs or crystals of NSAIDS at the mucosal surface. These features are the most important characteristic problems encountered in NSAID delivery.

This study was designed to investigate whether dendrimers might be able to act as drug carriers for NSAIDS and thus increase their aqueous solubility, reduce the local GI toxicity and control release of these drugs over a period of time. The controlled release of a drug is known as one of the methods to reduce GI toxicity (Florence & Jani 1994). Piroxicam and indomethacin were chosen as model NSAIDS. Both have a highly hydrophobic character and this makes them poorly soluble in water. Their structures are shown in Figure 6.1. The pKa of piroxicam and indomethacin are 6.3 and 4.5, respectively (Merck index 1996). Piroxicam and indomethacin are widely used and have considerable commercial importance in the treatment of pain, and the moderate to severe inflammation seen in rheumatoid arthritis and other musculoskeleton disorders. Indomethacin has been used to treat other indications including acute gout and dysmenorrhoea (British National Formulary 1997). As the lower gen anionic PAMAM gen (2.5 and gen 3.5) showed the highest rates of serosal transfer (Chapter 4) gen 2.5 was chosen as a model for these studies. As a reference control, PAMAM gen 3 was chosen as a cationic model as it has essentially the same stucture as gen 2.5 but a different surface.

In this study the following were investigated 1) The ability of PAMAM dendrimers to increase water solubility of piroxicam and indomethacin at various pHs 2) The acid-base properties of PAMAM dendrimers 3) The release of piroxicam and indomethacin from dendrimer complexes at different pHs. Various buffers were chosen for the study; hydrochloric acid buffer (pH 2), phosphate buffer (pH 6) and borate buffer



Piroxicam Mw 331.4



Indomethacin Mw 357.8

Figure 6.1. Structures of nonsteroidal anti-inflammatory model drugs

(pH 8) in order to cover the physiological pH range encountered from the stomach to the colon.

6.2. Methods

Solubility studies

The solubility of piroxicam in the presence of PAMAM dendrimers gen 2.5 and gen 3 was studied at three different pHs using the method described in section 2.2.10. The effect of PAMAM dendrimer gen 2.5, 3.5 and 5.5 or phosphate buffers (pH 6.0 - 9.0) and borate buffers (pH 8.0 - 10.0) on piroxicam solubility was also studied. Experiments to investigate the solubility of indomethacin in the presence of PAMAM dendrimers were also performed as above for piroxicam, except that in this case experiments were not undertaken at pH 8 due to the instability of drug in alkaline pH (British Pharmacopeia 1998).

The ability of PAMAM dendrimers to enhance the solubility of napthalene was also determined as described in section 2.2.10.

Acid-base properties of PAMAM dendrimers

PAMAM dendrimers with a carboxylate surface gen 2.5 and 3.5 and with an amine surface gen 3 and 4 were subjected to titration using HCl (1.07 M) as described in section 2.2.11. Titration curves were constructed by plotting the volume of acid added against pH, and from there, the number of charges per mole of dendrimer at different pH were calculated using the equations explained below.

Electrical neutrality in solution requires that the sum of all positive charges be equal to the sum of all the negative charges (Glasstone & Lewis 1960; Guenther 1975). In other words, the sum of negative charges and positive charges equal zero, so that the charge balance at each pH of titration in the case of PAMAM dendrimers gen 3 and gen 4 is shown in equation 6.1.

$$[X] + [H^+] + [Cl^-] + [OH^-] = 0 \qquad (6.1)$$

For gen 2.5 and 3.5 which have sodium carboxylate surface, equation 6.2 was used.

$$[X] + [H^+] + [Na^+] + [Cl^-] + [OH^-] = 0 \qquad (6.2)$$

where $[X]$ is the concentration of charge on dendrimer in mol/L

$[H^+]$ is the concentration of H^+ in mol/L as determined by the pH of the solution, $[H^+] = 10^{-pH}$

$[OH^-]$ is the concentration of OH^- in mol/L , $[OH^-] = 10^{(-pKw + pH)}$

$[C_{Cl^-}]$ is the concentration of Cl^- in mol/L, $[C_{Cl^-}] = [[HCl]V_{HCl}] / V_{tot}$

$[Na^+]$ is the concentration of Na^+ in mol/L, $[Na^+] = [[D]_i * N * V_i] / V_{tot}$

(where [HCl] is the concentration of HCl, V_{HCl} is the volume of HCl, V_{tot} is the total volume of solution, $[D]_i$ is the initial concentration of dendrimer, N is the theoretical number of Na^+ which is equivalent to the number of surface groups and V_i is the initial volume of dendrimer solution)

After the concentration of charge on dendrimer [X] was calculated, the number of charges per mole of dendrimer (θ) can be obtained using the following equation

$$\theta = [X] / [D] \qquad (6.3)$$

where [D] is the concentration of dendrimer, $[D] = [[D]_i V_i] / V_{tot}$

The titration plots: number of charged species/ dendrimer against pH were plotted and the pKa and end points of titration were obtained from these graphs. Schematic diagrams showing the structure of dendrimers with different degrees of protonation were also prepared to explain the changes.

Release of drugs from PAMAM dendrimers complexes

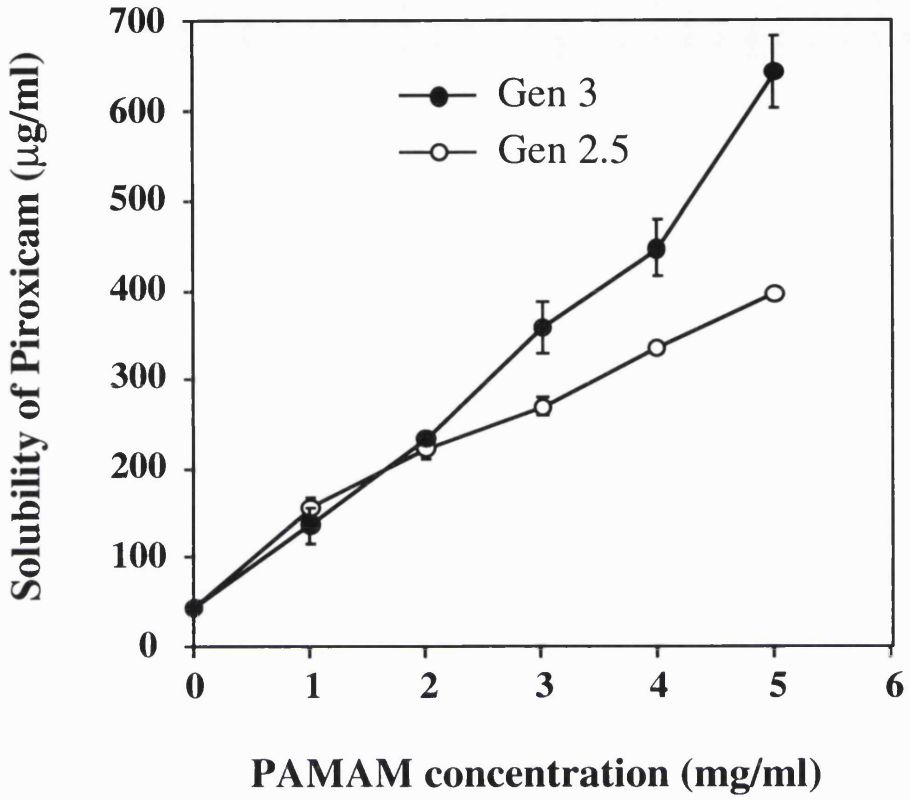
Complexes of PAMAM dendrimers with piroxicam or indomethacin were prepared as described in section 2.2.12. To study the rate of drug liberation, each sample was put into a dialysis tube Mw cut-off 2,000 Da and the amount of drug released into the buffers at pH 6 and 8 were measured using the dialysis method described in section 2.2.12.

6.3. Results

6.3.1. Solubility of piroxicam in the presence of dendrimers

The solubility of piroxicam at pH 6 and pH 8 was found to increase with the addition of PAMAM dendrimer (Figure 6.2). When piroxicam was mixed gen 2.5 solubility was approximately 8.9 fold greater than seen for piroxicam alone and with addition of gen 3 the solubility was increased 9.7 fold. At pH 8 the solubility of piroxicam increased 2 and 5.4 fold with addition of gen 2.5 and gen 3, respectively. Improved solubility was not seen at pH 2 (data not shown). The UV spectrum of piroxicam measured in the presence of various concentrations of gen 2.5 and 3 dendrimer showed no spectral shift or change in λ_{max} (Figure 6.3). Also the UV spectra of

(a)



(b)

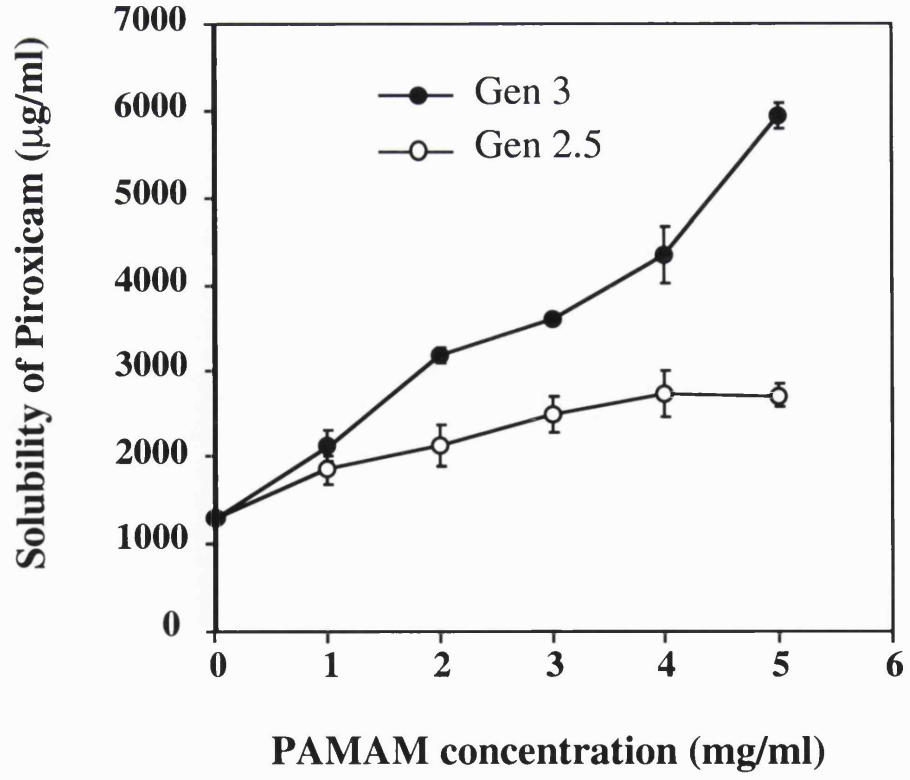
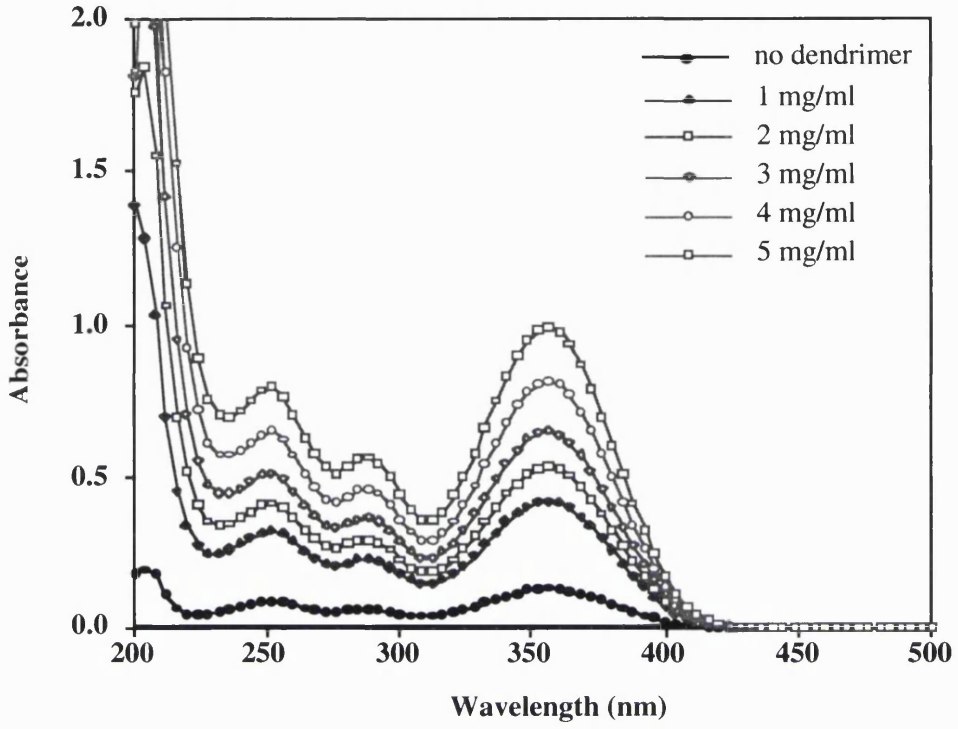


Figure 6.2. Effect of PAMAM dendrimers on the solubility of piroxicam in (a) buffer pH 6 and (b) pH 8, mean (n = 3) ± S.D.

(a)



(b)

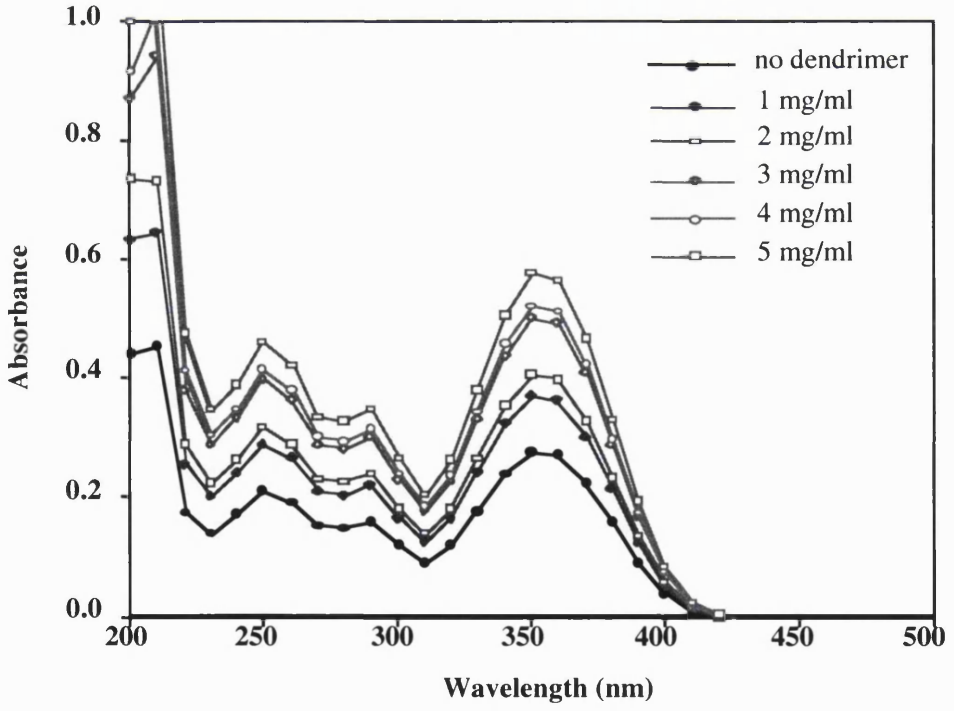


Figure 6.3. The spectra of piroxicam in various concentration of dendrimer gen 2.5 at (a) pH 6 and (b) pH 8

dendrimer alone showed no absorbance at the λ_{max} used to measure the drug concentration (Figure 6.4).

6.3.2. Effect of pH and buffer on piroxicam solubility

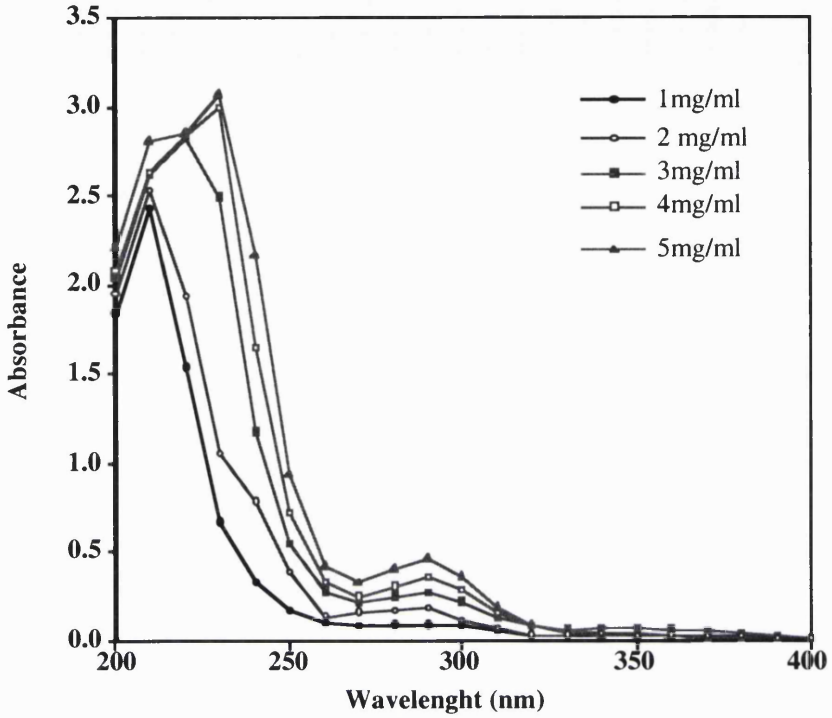
The effect of pH on the solubility of piroxicam in phosphate and borate buffer solution are shown in Figure 6.5. In case of phosphate buffer solution, the solubility of piroxicam increased ~ 7 fold from pH 6 to 7, ~ 4 fold from pH 7 to 8 and ~ 2 fold from pH 8 to 9. When borate buffer was used, the solubility of piroxicam increased ~ 2.5 times from pH 8 to 9 and ~ 2 times from pH 9 to 10.

Due to the high basicity of PAMAM dendrimers, they can alter the pH of buffers into which they are placed. After adding PAMAM dendrimer (up to 5 mg/ml) to phosphate buffer, the pH increased by ~ 1 unit (from 6 to 7). Addition of PAMAM dendrimer to borate buffer caused a 0.5 unit increase (from 8 to 8.5) (data not shown). To find out whether the enhancement in piroxicam solubility reported in section 6.3.1 was simply due to this pH effect alone, the solubility of piroxicam in different buffers with or without dendrimer was compared (Figure 6.6). In the case of PAMAM gen 2.5, it can be seen that at the highest dendrimer concentration used (5 mg/ml) of dendrimer there was no significant ($p < 0.05$) enhancement in drug solubility due to the presence of dendrimer. This was also true in the phosphate and borate buffers. In contrast, enhancement of drug solubility after addition of dendrimer gen 3 was higher than could be attributed to pH change in buffer alone. The dendrimer effect was more pronounced when the pH of the mixing solution was more than 6.3 (this is the pKa of piroxicam) and when the concentration of dendrimer was increased. The effect of different generations of carboxylate dendrimer (gen 2.5, 3.5 and 5.5) on piroxicam solubility in buffer pH 8 is shown in Figure 6.7. Generally there was no significant difference in solubility relating to dendrimer gen except at high concentration, where gen 5.5 showed an enhancement of solubility that was higher than seen for gen 2.5 and gen 3.5.

6.3.3. Solubility of indomethacin in the presence of dendrimers

The solubility of indomethacin at pH 6 increased with the addition of PAMAM dendrimers (Figure 6.8). Gen 2.5 caused ~ 5 fold increase and gen 3 a 10 fold increase. The improved of the solubility was also not seen at pH 2 (data not shown). The UV spectrum of indomethacin seen with addition of various concentrations of PAMAM dendrimer gen 2.5 and 3 showed no spectral shift or change in λ_{max} (Figure 6.9).

(a)



(b)

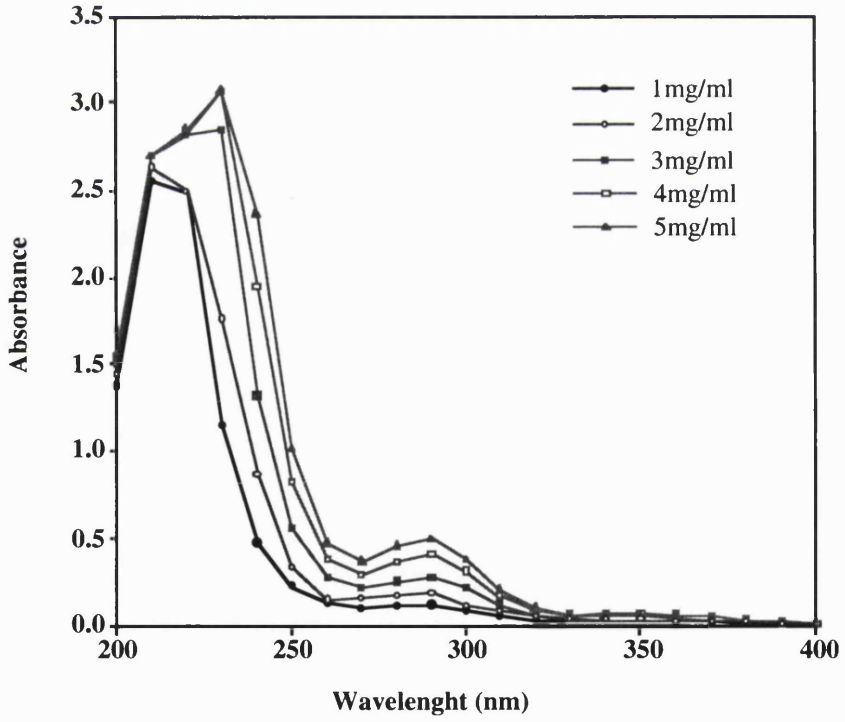
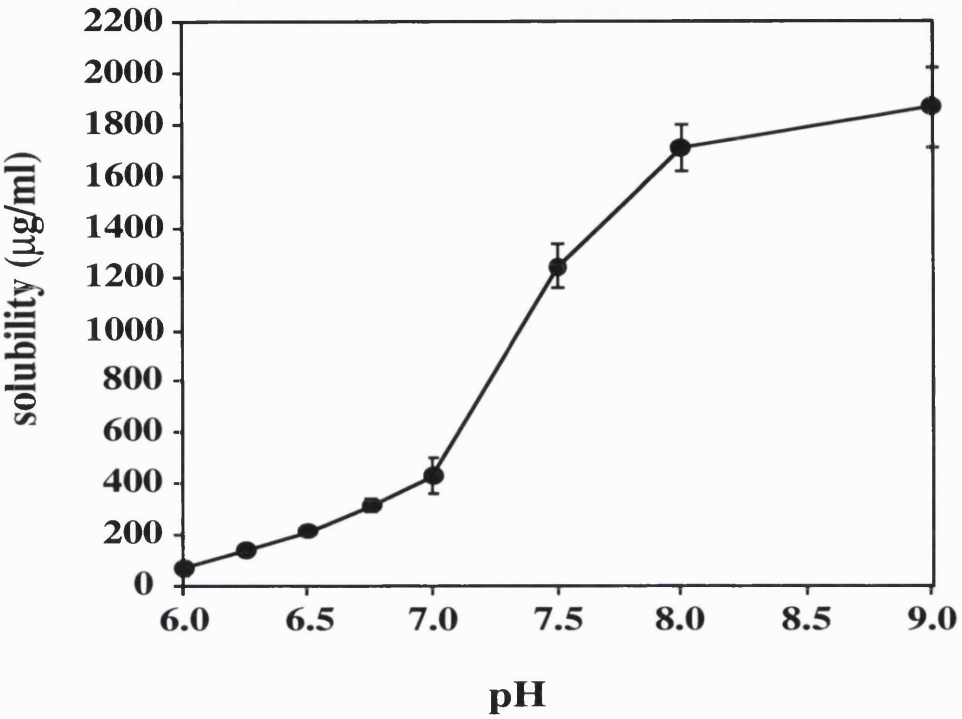


Figure 6.4. The spectra of PAMAM dendrimer alone in buffer pH 6 (a) gen 2.5 and (b) gen 3

(a)



(b)

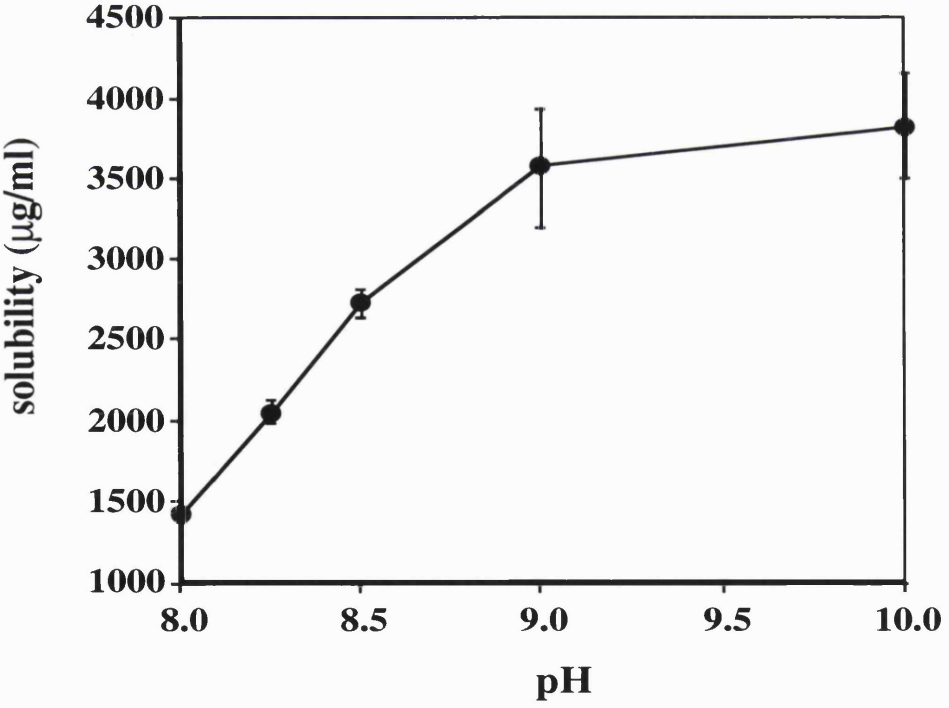
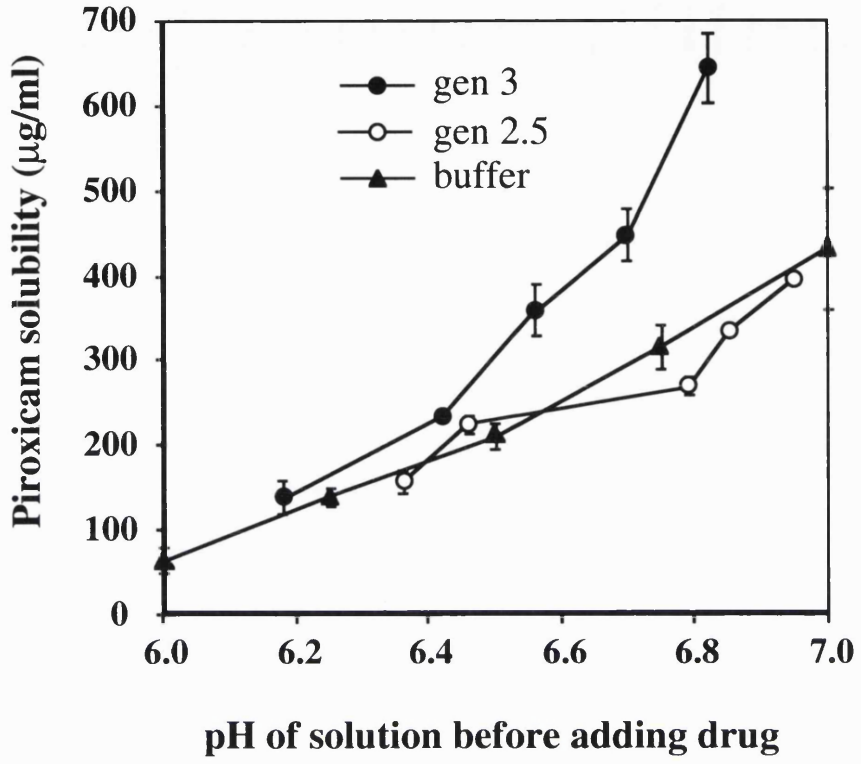


Figure 6.5. Effect of pH on the solubility of piroxicam in (a) phosphate buffer and (b) borate buffer, mean (n = 3) ± S.D.

(a)



(b)

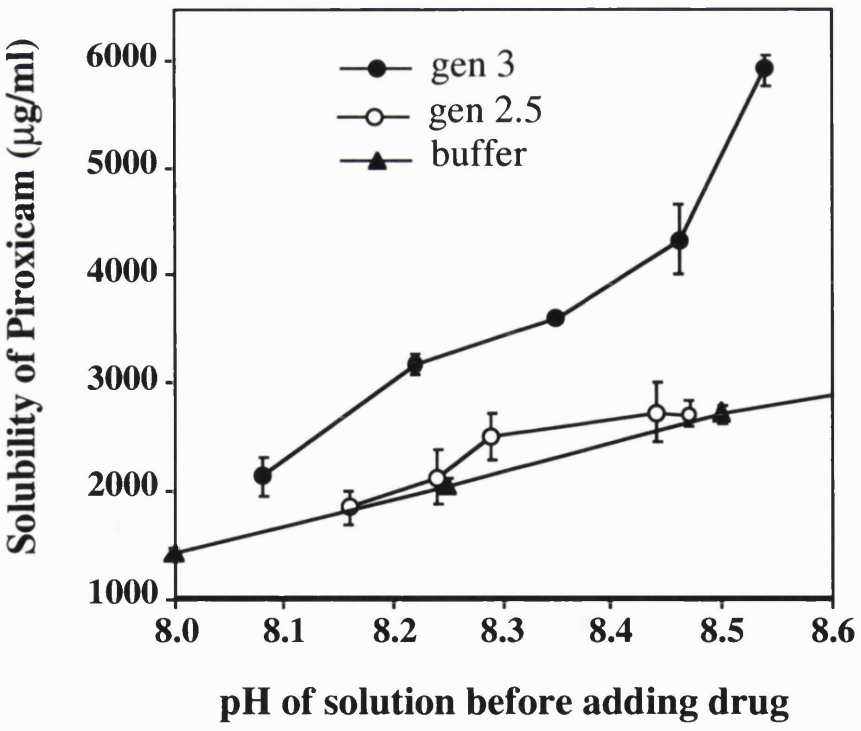


Figure 6.6. Solubility of piroxicam in different pH of buffer solution alone and buffer solution with dendrimer (a) phosphate buffer (b) borate buffer, mean (n = 3) ± S.D.

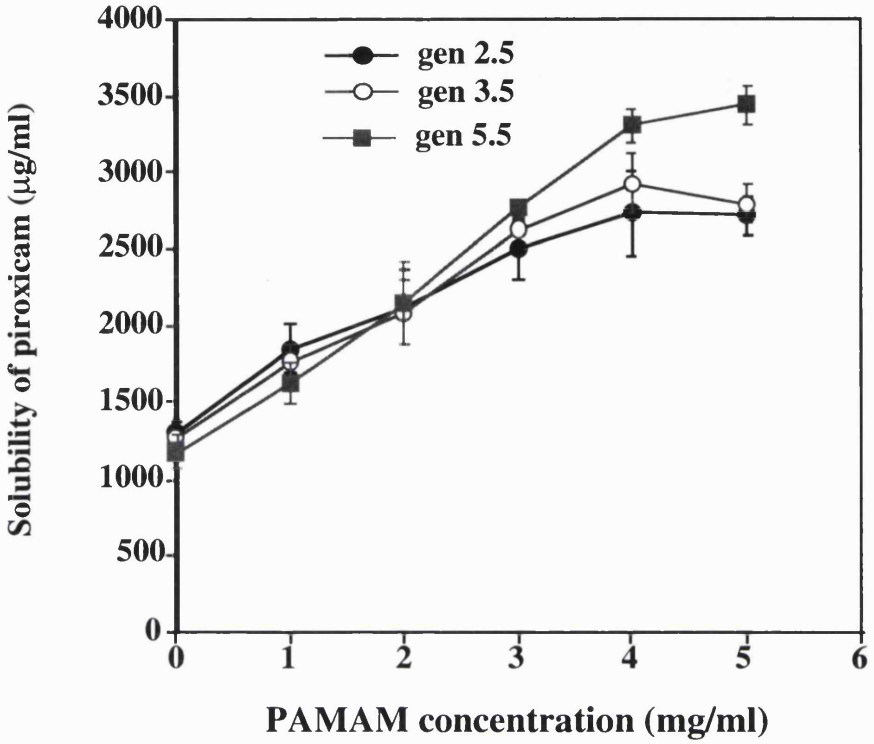


Figure 6.7. Effect of different gen on the solubility of piroxicam at pH 8, mean (n = 3) ± SD

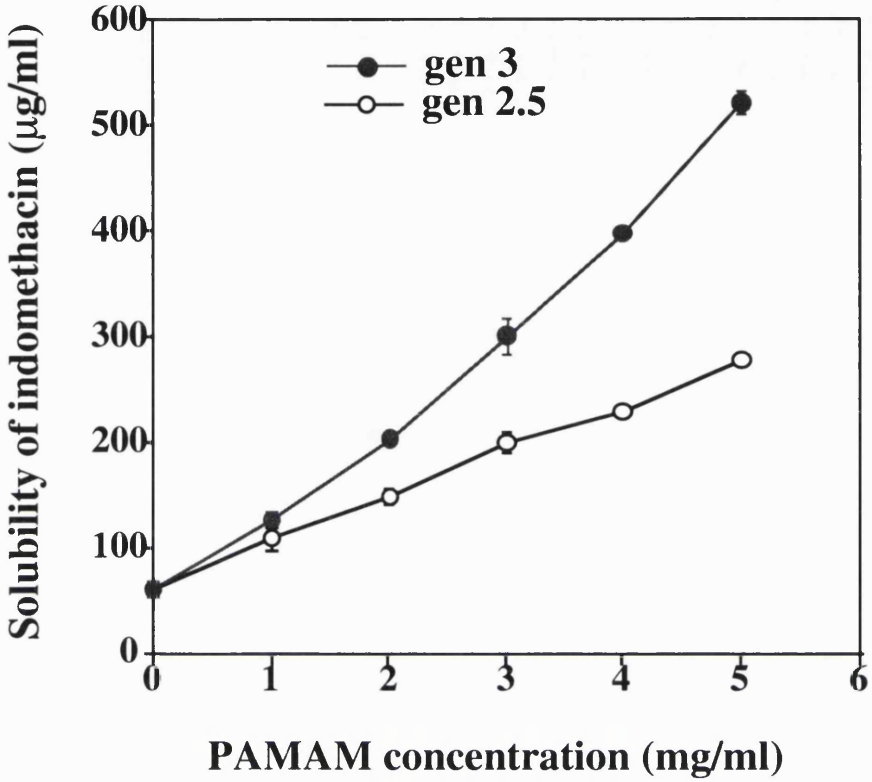


Figure 6.8. Effect of PAMAM dendrimers on the solubility of indomethacin in buffer pH 6, mean (n = 3) ± SD

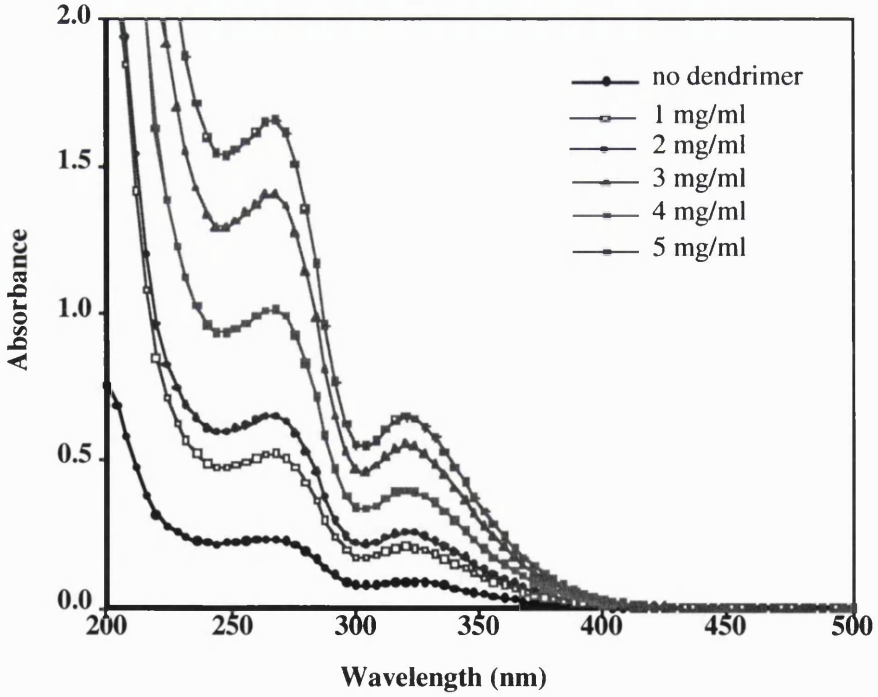


Figure 6.9. The spectra of indomethacin in various concentration of PAMAM dendrimer gen 3

6.3.4. Effect of pH and buffer on indomethacin solubility

Changes in buffer pH also influenced the solubility of indomethacin. In phosphate buffer (Figure 6.10) drug solubility increased ~ 12 fold with an increase pH from 6 to 7. To find out whether the enhancement in drug solubility seen in the presence of dendrimer was due to the pH effect alone, indomethacin solubility in buffer with and without dendrimers was compared (Figure 6.11). There was no significant difference ($p < 0.05$) in drug solubility in the absence or presence of dendrimer gen 2.5 at the highest concentration used. However, a significant increase in solubility ($p < 0.05$) was seen when gen 3 was added to indomethacin, although the increase solubility was not as high as that seen for piroxicam.

6.3.5. Enhancement of the solubility of napthalene by dendrimers

Preliminary experiments to investigate the solubility of the hydrophobic molecule, napthalene, in the presence of dendrimers showed no significant difference ($p < 0.05$) between the solubility of napthalene in water and napthalene in water in the presence of dendrimer gen 2.5 or gen 3 (Table 6.1).

6.3.6. Acid-base properties of PAMAM dendrimers

Similar titration curves were obtained for PAMAM gen 3 and gen 4 (Figure 6.12 - 6.13) and for PAMAM gen 2.5 and 3.5 (Figure 6.14 - 6.15). The titration curves obtained for all PAMAM dendrimers indicated that protonation gives 2 rather sharp breaks which are endpoints of protonation.

The relationship between the estimated number of charged groups (protonation) and pH are shown in Figure 6.16 - 6.19 and the pK_a and the end points of protonation estimated from these curves are summarised in Table 6.2. The pK_a for terminal amine groups and interior tertiary amine groups for gen 3 and gen 4 were estimated to be in the range of 9.2 - 9.5 and 6.1 - 6.2, respectively. The number of protonated groups estimated after complete titration were compared with the theoretical number of groups available for each molecule in Table 6.3. The estimated number of protonated groups per mole for gen 3 and 4 are in good agreement with the known dendrimer structure (Table 6.3). For example, gen 3 contains 32 terminal primary amine groups and 30 internal tertiary amine groups. The protonation curve indicated that approximately 64 groups were charged at the end point.

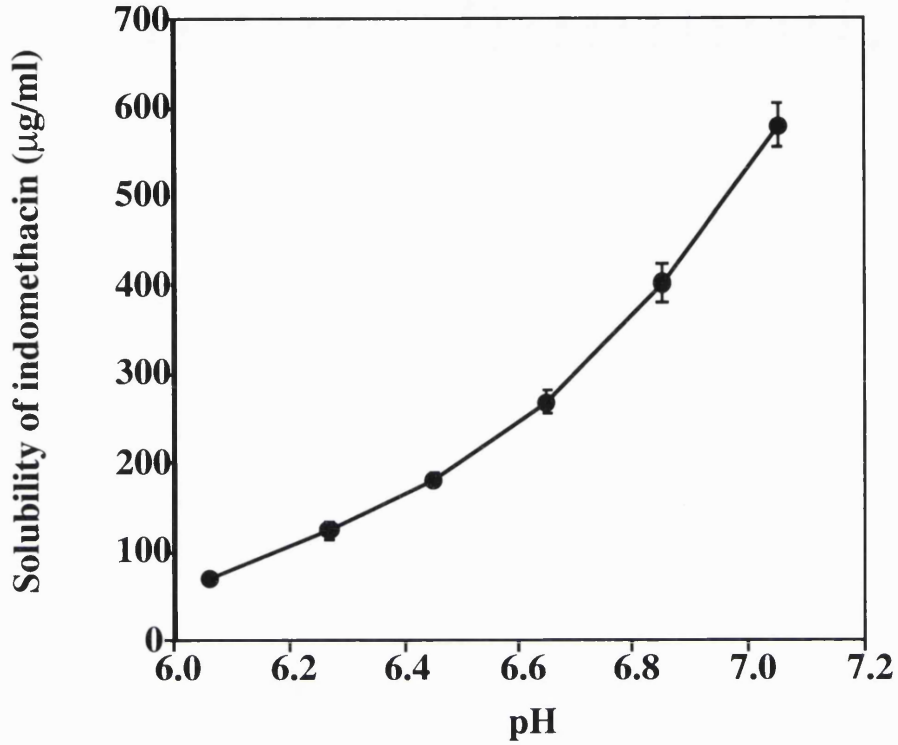


Figure 6.10. Effect of pH on the solubility of indomethacin in phosphate buffer, mean (n = 3) ± S.D.

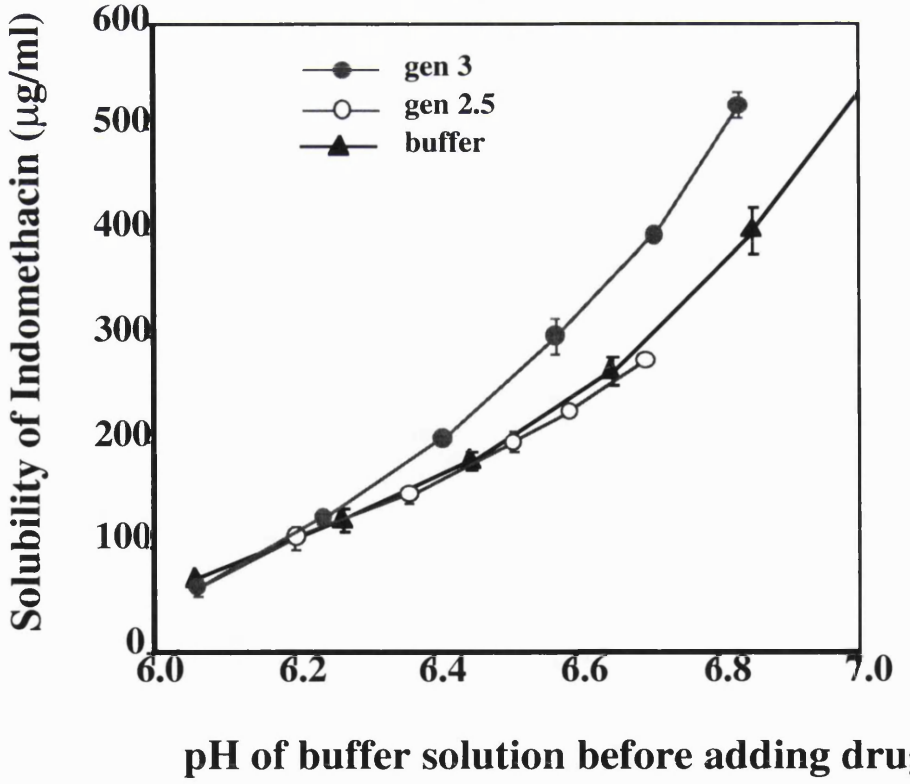


Figure 6.11. Solubility of indomethacin in different pH of buffer solution alone and buffer solution with dendrimer, mean (n = 3) ± S.D.

Table 6.1. Solubility of napthalene in water with and without dendrimer after sonication for 3 h, mean (n = 3) ± S.D.

	Solubility (μg/ml)
napthalene	1.96 ± 0.53
napthalene with gen 2.5	2.18 ± 0.36
napthalne with gen 3	2.09 ± 0.77

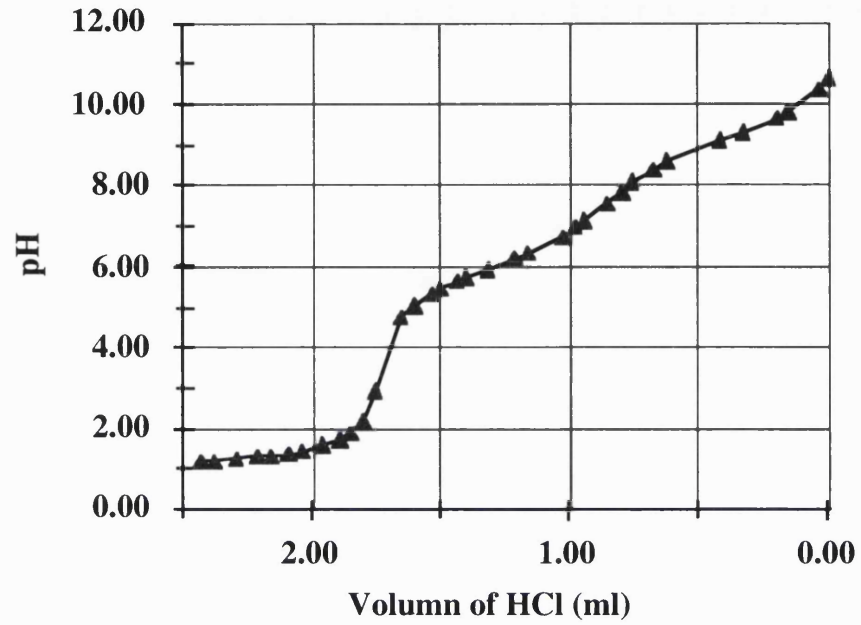


Figure 6.12. Titration curve of PAMAM dendrimer gen 3

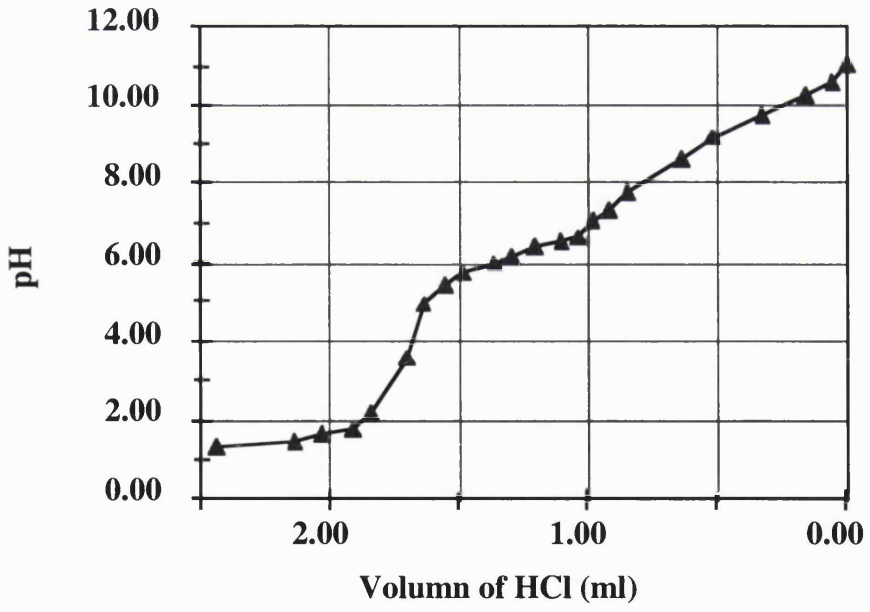


Figure 6.13. Titration curve of PAMAM dendrimer gen 4

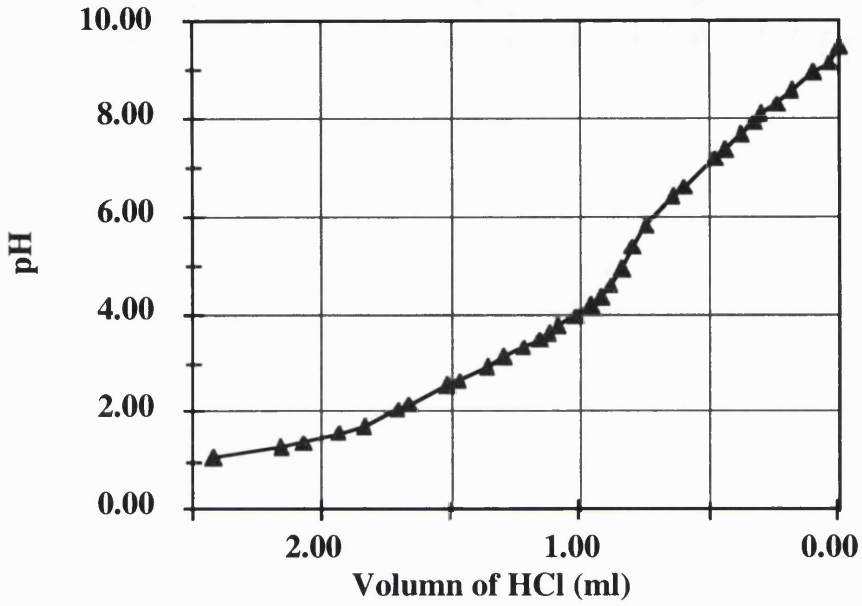


Figure 6.14. Titration curve of PAMAM dendrimer gen 2.5

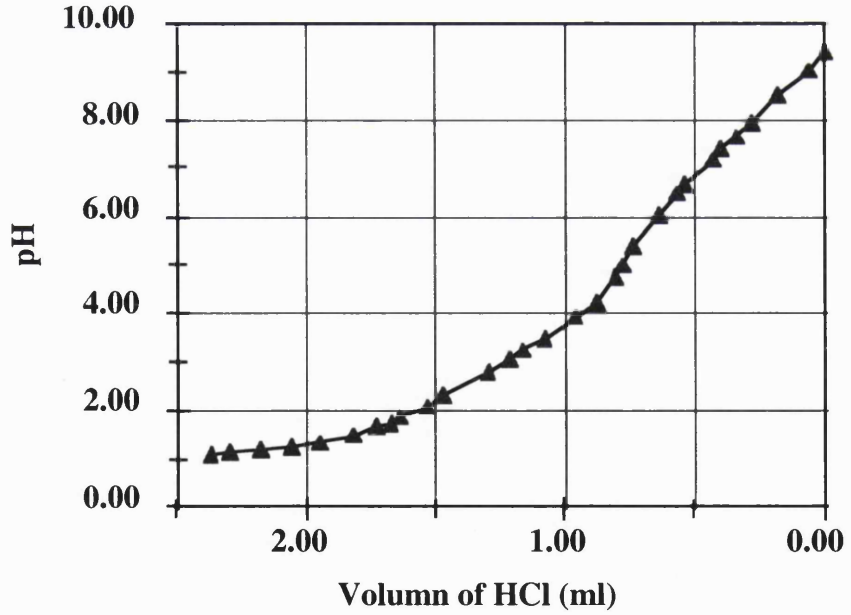


Figure 6.15. Titration curve of PAMAM dendrimer gen 3.5

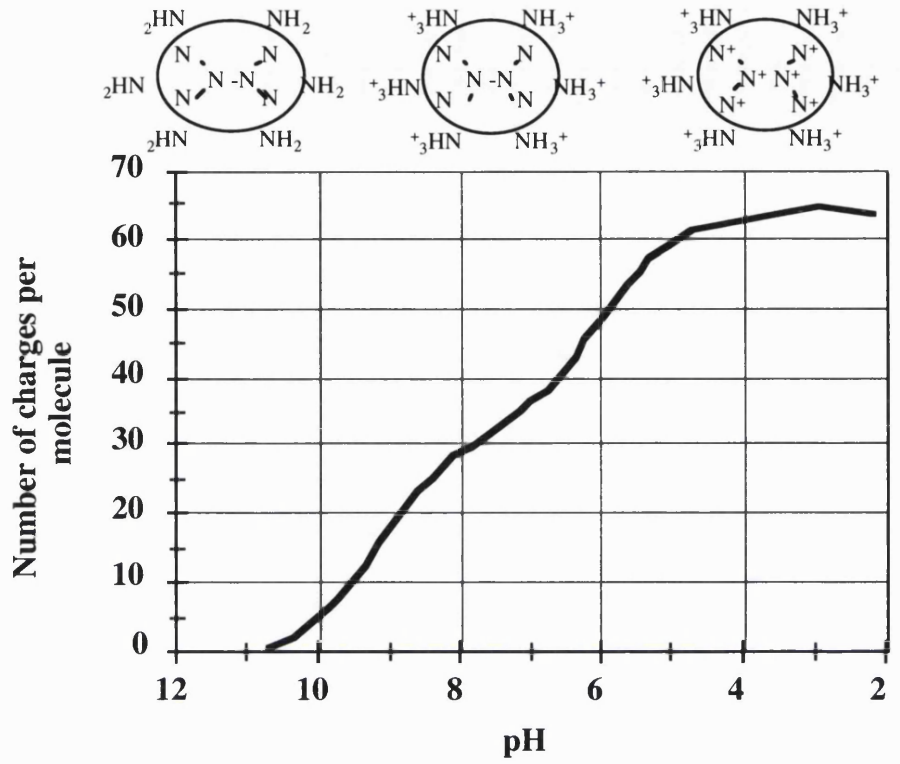


Figure 6.16. Effect of pH on charge characteristic of PAMAM dendrimer gen 3

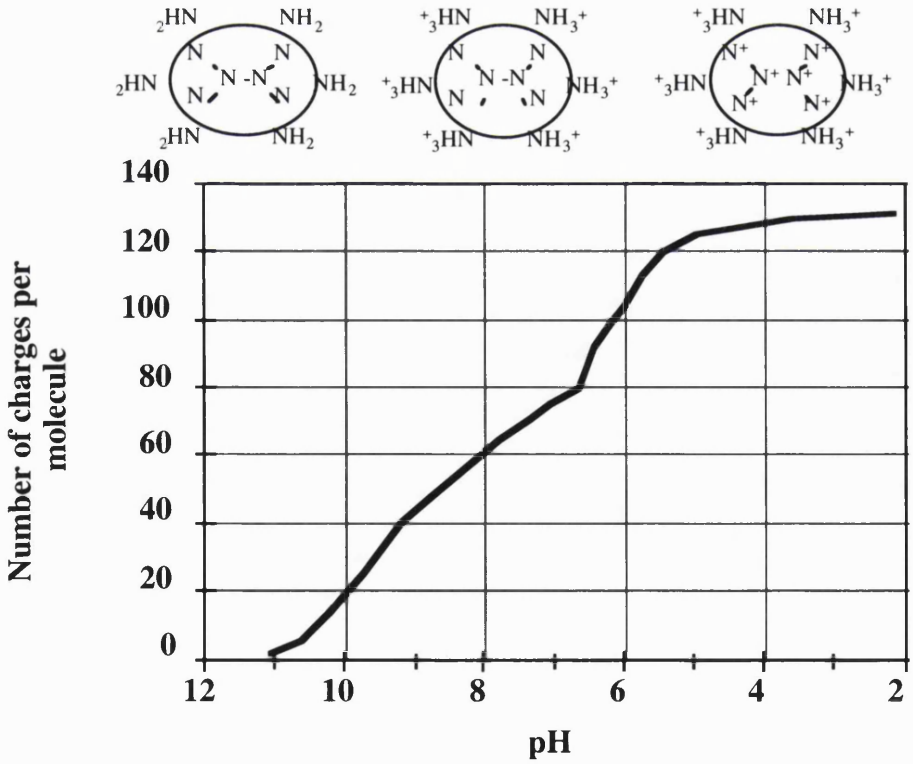


Figure 6.17. Effect of pH on charge characteristic of PAMAM dendrimer gen 4

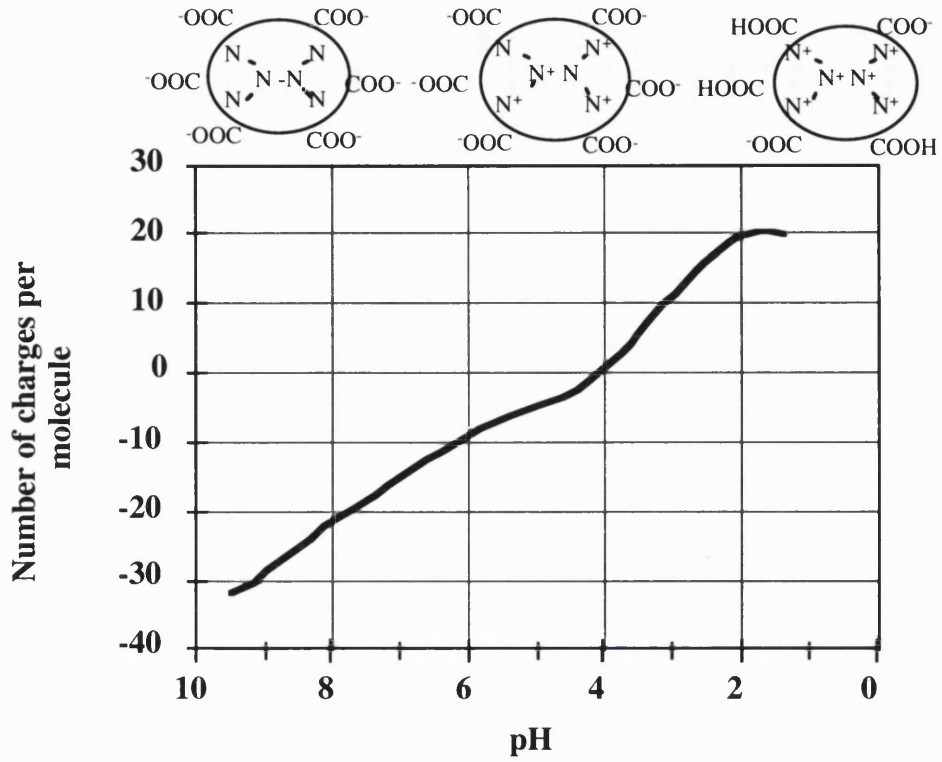


Figure 6.18. Effect of pH on charge characteristic of PAMAM dendrimer gen 2.5

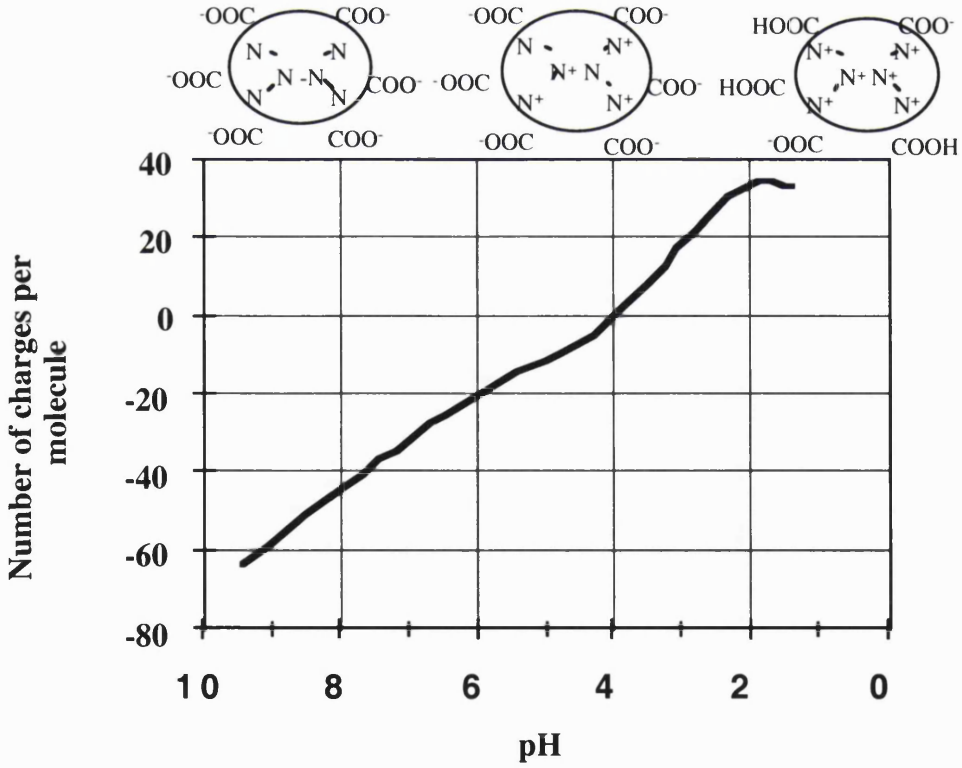


Figure 6.19. Effect of pH on charge characteristic of PAMAM dendrimer gen 3.5

Table 6.2. pH titration of the PAMAM dendrimers

PAMAM dendrimers	pKa		pH at the end point		Possible groups of protonation	
	1st pKa	2nd pKa	1st end point	2nd end point		
gen 1 (Tomalia 1990)	9 - 10	4 - 5	~ 6.85	~ 3.86	1° amine (surface)	3° amine (interior)
gen 3	~ 9.2	~ 6.1	~ 7.5	~ 3.4	1° amine (surface)	3° amine (interior)
gen 4	~ 9.5	~ 6.2	~ 7.7	~ 3.6	1° amine (surface)	3° amine (interior)
gen 2.5	~ 7.0	*	~ 4.0	~ 2.0	3° amine (interior)	
gen 3.5	~ 6.9	*	~ 4.0	~ 2.0	3° amine (interior	

* The value cannot be estimated due to uncomplete protonation

The sequence of protonation of gen 3 is proposed in Figure 6.12.

1) At pH ~ 9.2, the first pK_a value, approximately half of the surface primary amine groups are protonated.
2) At pH ~ 7.5 protonation of the surface groups is completed.
3) At pH ~ 6.1, the second pK_a value, approximately half of the internal tertiary amine groups are protonated.
4) Finally, at pH ~ 3.4 the protonation of the internal tertiary amine is completed.

The protonation of gen 4 can be described in a similar way except that the number of groups available for protonation is higher in this case (Table 6.3). For gen 4 the estimated of species available for protonation was closer to that of the proposed structure.

Due to the presence of carboxylate surface groups in PAMAM gen 2.5 and gen 3.5, the interior tertiary amine groups (pKa ~ 9-10) are more likely to be protonated first due to their stronger basicity than the surface carboxylate groups (pKa < 5) (March 1992) and the observed end points of this titration are shown in Table 6.2. In the case of gen 2.5 and 3.5 the number of total protonated groups that were estimated were less than the number of groups theoretically available in dendrimer molecule (77% of the total were protonated) (Table 6.3). The estimated pK_a for gen 2.5 and 3.5 are in the range of 6.9 - 7.0. The possible explainations for protonation of gen 2.5 and gen 3.5 (Figure 6.18 - 6.19) are:

1) At the beginning all of carboxylate groups are negatively charged.
2) At pH ~ 4 most of the interior tertiary amine are protonated and the net charge of molecule is zero.
3) Finally, at pH ~2 some of the surface carboxylate groups become protonated.

6.3.7. Drug release studies

The release of piroxicam and indomethacin from PAMAM gen 2.5 and 3 complexes is shown in Figures 6.20 and 6.22. No significant difference was observed between the rates of transfer of drug alone out of the dialysis bag compared with that observed for dendrimer complexes ($p < 0.05$). About 50% of the drug was released over 30 min and all of the drug was released within 2 h.

6.4. Discussion

These experiments used a simple procedure to study complexation of drugs with PAMAM dendrimers in aqueous solution. The solubility enhancement of piroxicam and

Table 6.3. Number of protonated groups after titration compared to the number of theoretical groups available

PAMAM dendrimers	Number of groups available for protonation		Number of total protonated groups
	surface	interior	
gen 3	32 (1° amine)	30 (3° amine)	64
gen 4	64 (1° amine)	62 (3° amine)	130
gen 2.5	32 (carboxylate)	30 (3° amine)	48
gen 3.5	64 (carboxylate)	62 (3° amine)	98

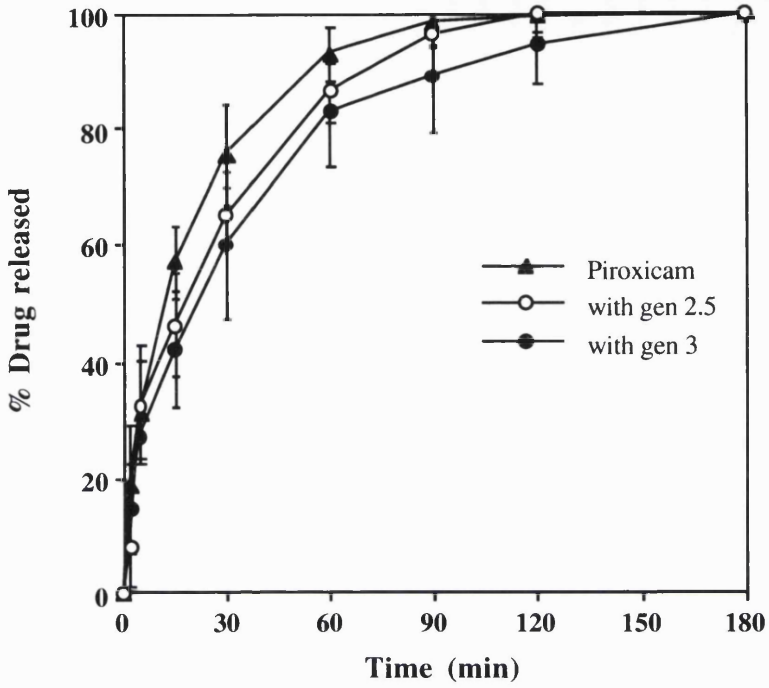


Figure 6.20. Percent Piroxicam released in buffer pH 6 from the system with and without dendrimer, mean (n = 3) ± S.D.

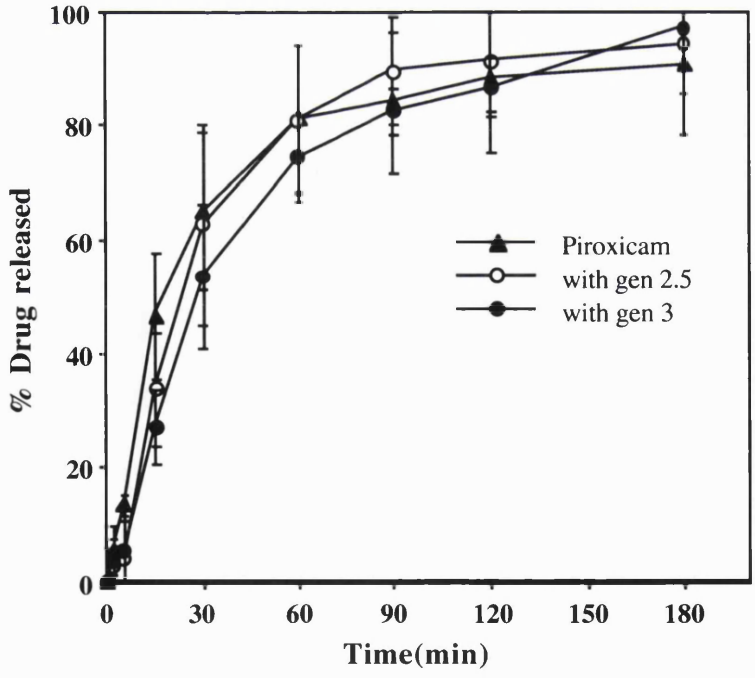


Figure 6.21. Percent piroxicam released in buffer pH 8 from the system with and without dendrimer, mean (n = 3) ± S.D.

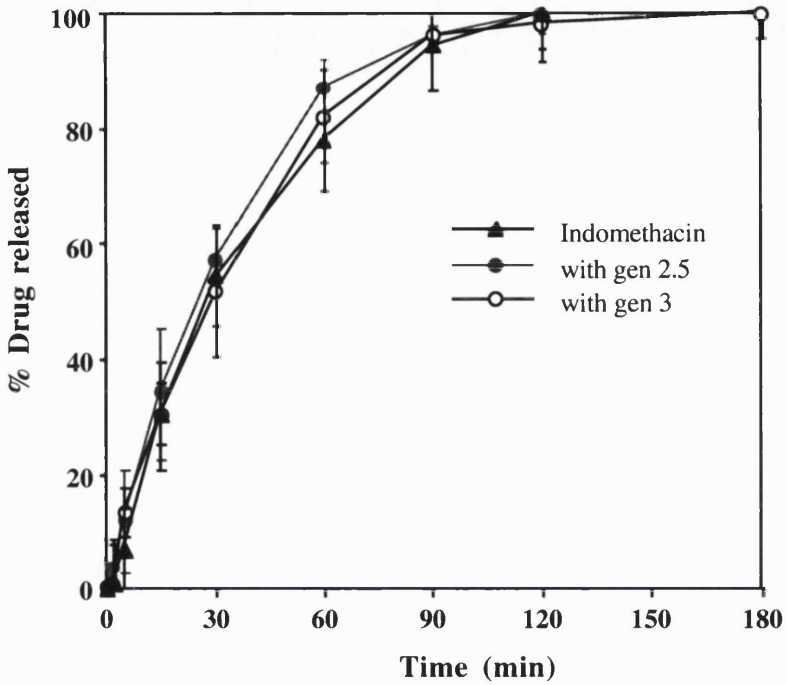


Figure 6.22. Percent indomethacin released in buffer pH 6 from the system with and without dendrimer, mean (n = 3) ± S.D.

indomethacin was largely due to the ability of PAMAM dendrimers to alter the buffer pH. As the pH of buffer solution was increased above the pKa value of each drug (6.3 for piroxicam and 4.5 for indomethacin) the solubility increased dramatically due to the increasing ionisation of the drug molecules. In contrast, at pH 2 (below pKa of both drugs) piroxicam and indomethacin were almost insoluble and addition of dendrimer did nothing to increase solubility. This was because there was no drug being available to be complexed with the dendrimers and the results also indicate there was no hydrophobic interaction occuring between drug molecule and the dendrimer interior.

The simple observations made here demonstrate the need to take the basicity of the environment into account once dendrimers with carboxylate or amine groups on the surface are added. In contrast, PAMAM dendrimers with hydroxy terminal groups have been reported to improve the solubility of many acidic compounds (e.g. benzoic acid, 1-napthoic acid, 9-anthracenecarboxylic acid) regardless of their pH effect (Esfand et al 1996). Although PAMAM gen 2.5 increased the solubility of piroxicam simply due to its ability to change media pH, dendrimer gen 3 did demonstrate some interaction with piroxicam leading to an increase in solubility in addition to pH effect. At both pH 6 and 8 this effect was more pronounced when the concentration of dendrimer was increased. Although in the case of indomethacin, PAMAM dendrimer gen 3 also showed some interaction with the drug at pH 6, the difference in solubility from gen 2.5 was not as high as seen for piroxicam. Perhaps not surprisingly this suggests that the the structure and physical properties of each drug has different effect on the interaction with the dendrimer.

It is interesting to discuss the possible mechanisms of dendrimer-drug interaction. PAMAM dendrimer gen 2.5 and gen 3 have the same core structure composed of internal tertiary amines and amide groups. They only differ at the surface as gen 2.5 contains carboxylate and gen 3 contains primary amine groups. Analyses of dendrimer acid-base behaviour using a titration method gave a better understanding of the charge characteristics of these complicated molecules. The numerous interior and exterior groups contained within the dendrimer structure have different charges at different pH.

In the case of PAMAM dendrimer gen 3, at the experimental pH of 6-7, all the surface primary amine groups and the limit number of the internal tertiary amine groups are protonated (Fig. 6.16). At the same time, the number of ionised drug molecules would also increase with an increase in the pH of the buffer solutions used (above the drug pKa). It is therefore likely that the protonated surface amines of the PAMAM dendrimer gen 3 molecule could interact electrostatically with the negatively charged group of piroxicam. This interpretation is in good agreement with the recent results of

Milhem et al (1999) who showed that PAMAM dendrimer gen 4 can increase the solubility of ibuprofen due to specific electrostatic interactions between the carboxyl groups of drug molecules and the surface amines on dendrimer. Furthermore, interaction studies using salicylic acid and PAMAM dendrimers demonstrated a charge interaction between drug and full gen PAMAM (gen 4) but not the half gen (gen 4.5) and that a greater extent of interaction occured at pH 5 than at pH 6.65 due to the higher percentage of dendrimer ionization (Tomalia 1995).

Although for gen 3 the number of the protonated surface amine present at pH 8 - 8.6 would be less than seen at pH 6, dendrimer gen 3 was still able to enhance the solubility of piroxicam and the effect was even more pronounced at pH 8 - 8.6 than at lower pH. Therefore, it is likely that other types of drug-dendrimer interaction were responsible. The interior of PAMAM dendrimers has been described by some researchers as a "hydrophobic cavity" which facilitates the incorporation of hydrophobic molecules (Esfand et al 1996, Tomalia et al. 1998). The term "inclusion complexes" has been proposed to describe the enclosed molecule or guest entrapped inside the dendrimer. However, as the PAMAM gen 3 showed no enhancement in the solubility of naphthalene (a very hydrophobic compound) this hydrophobic entrapment theory cannot be the explaination of this case.

On the other hand, the interior of PAMAM dendrimers contains numerous tertiary amine and amide group which are potential hydrogen-bonding sites. Dopamine, which possesses both hydrogen-bond donor and acceptor sites, has been shown by use of molecular dynamics calculations to hydrogen bond with the PAMAM dendrimer's interior carbonyl, amide and amine groups (Tomalia et al. 1990). The piroxicam molecule contains many groups capable of hydrogen bondings $-SO_2$, $-N-CH_3$ group, -C= O group, -N-H group and pyridine-N_2 and it has preferential Lewis base property (better proton acceptor) (Jain et al. 1997; Bustamante et al. 1998). Thus it is possible that hydrogen bonds may be formed between piroxicam and the PAMAM interior amide groups. In comparison, the indomethacin molecule would not be capable of forming hydrogen bonds with the dendrimers interior so indomethacin could interact only by electrostatic interaction. Further investigations using microcalorimetry (Ehtezazi et al 1999) or ^1H NMR titration (Newkome et al 1996) would be interesting to try to elucidate the type and the extent of interaction further.

Whatever the exact mechanisms of complexation, the release studies showed conclusively that the interaction was not strong enough to prevent drug easily diffusing out of the dendrimer host depending on the equilibrium conditions. As this study intended to investigate PAMAM dendrimer as oral drug delivery systems there is a need

for a more controllable and stable interaction between the drug and dendrimer than was observed here for the dendrimer-drug complexes. Synthesis of covalent dendrimer-drug conjugates provides an opportunity to design in there features so a covalent anti-inflammatory agent-dendrimer conjugate was prepared and this is described in Chapter 7.

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Chapter Seven

Synthesis and characterisation of PAMAM dendrimer-ibuprofen conjugate: A preliminary study

7.1. Introduction

As the solubility of model NSAIDS: piroxicam and indomethacin was improved either by complexation with dendrimers and/or pH effect, but the release of drug from the system was too rapid (Chapter 6), it was decided to synthesise drug-dendrimer conjugates using a covalent linkage for drug attachment. The purpose of synthesising this conjugate was both to improve drug solubility whilst giving better control of drug release at the target site. Optimisation of the dendrimer-drug linker could subsequently introduce the possibility to control of rate and site of drug release.

In this study ibuprofen and PAMAM dendrimer gen 3 were used as a preliminary model drug-dendrimer conjugate. Their chemistry allows simple conjugation by reaction of the drug's carboxylic group with the amine surface of the dendrimer. Ibuprofen is one of the most widely used NSAID for treatment of pain and arthritis (Davies 1998). The drug has a low water solubility and pKa value of 5.2. Although cationic PAMAM dendrimers were shown to be toxic to the cells and the intestinal tissue at high concentration (Chapter 3 and Malik et al. 1999) it was hoped that a safe concentration of dendrimer could be used if the drug loading of the dendrimer conjugate was high enough. Alternatively the chemistry could in future, be modified to all use of the anionic PAMAM or other dendrimers.

Development of a controlled release delivery systems for anti-inflammatory drugs such as ibuprofen which have short half-life ($t_{1/2}$ = 2 h) (Dollery 1999) would be particularly useful for the treatment of rheumatoid arthritis. The symptoms associated with rheumatoid arthritis display a circadian rhythm and the maximal effect of an evening dose of painkiller is actually needed the following morning. Many studies have been undertaken to obtain nonsteroidal anti-inflammatory controlled-release systems, such as tablets, hard gelatin capsules, coated pellets, oral suspensions and microspheres or microcapsules (Table 7.1).

In addition in recent years much attention has focused on the development of prodrugs of NSAIDS that can reduce GI related side-effects. It is known that GI irritation occurs after long-term exposure to NSAIDS. Use of prodrugs to temporarily mask the acidic group of NSAIDS has been shown to significantly reduce GI toxicity. Most of the NSAIDS prodrugs synthesised have been in ester forms for example, alkyl ester (Venuti et al. 1989), glycol amide ester (Bundgaard & Nielsen 1988), thioester (Venuti et al. 1989), morpholinoethyl esters (Tammara et al. 1993), triethylene glycol ester (Bonina et al. 1996).

Table 7.1. Examples of NSAIDS controlled release systems

System	Drug	References
Capsule	Ibuprofen	Kumar & Pandit 1997
Coated pellets	Ibuprofen	Singh et al. 1995
Microcapsules	Indomethacin	Tirkkonen et al. 1995
Microspheres	Ibuprofen	Tamilvanan & Sa 1999
	Indomethacin	Casteli et al. 1997
	Piroxicam	Lalla & Sapna 1993
Polymeric drug	Ibuprofen	Parejo et al. 1998
	Ketoprofen	Parejo et al. 1998
Suspension	Ibuprofen	Kawashima et al. 1991
Tablets	Ibuprofen	Palmieri et al. 1999
	Indomethacin	Xu et al. 1997

A dendrimer-drug conjugate could allow controlled release of the anti-inflammatory agents at a desired rate and this could also prevent the GI irritation, and in addition could also be useful in different applications depending on the site of drug release. For example in the small intestine, the dendrimer could act as a simple platform for either controlled drug release and thus promote absorption into the systemic circulation. In this context the delivery system may be useful for the treatment of rheumatoid arthritis. Alternatively the dendrimer conjugate could be designed to release drug further down the GI tract and thus to promote colon-specific delivery. Diseases of the colon such as irritable bowel syndrome, Crohn's disease and ulcerative colitis would be more effectively treated if the drug was applied directly to the affected area (Kalala et al. 1996). Local delivery of drugs is advantageous in itself, permitting lower dosing and resulting in fewer side-effects. Utilisation of azo-bond which would be cleaved by bacterial enzymes or the use of pH-dependent linkers are both methods normally used for targeted drug release in the colon (Ashford & Fell 1993). These approaches could easily be applied to a dendrimer-drug delivery system.

In this study, a water soluble PAMAM gen 3-ibuprofen conjugate was synthesised, the drug being attached to the dendrimer via amide bond using a carbodiimide (EDC) linker. The reaction scheme is shown in Figure 7.1. It should be noted that this model conjugate contains an amide bond linker which is not biodegradable. Therefore the compound would not be expected to be pharmacologically active, but was prepared only as a model which would be useful to monitor pharmacokinetics *in vitro* and *in vivo*.

7.2. Methods

The dendrimer-ibuprofen conjugate was synthesised according to the method described in Chapter 2 (section 2.2.2.5) using different ratios of dendrimer : ibuprofen. A small amount of [^{14}C]ibuprofen was also added to enable the reaction to be monitored and the conjugate characterised. The conjugate was purified and characterised by sephadex G-25 column chromatography as described in Chapter 2 (section 2.2.2.5). The % w/w of ibuprofen in the conjugate was determined by measuring the incorporation of radioactivity. The structure of dendrimer-drug conjugate was confirmed by ^{1}H NMR and the amount of free drug in the conjugate was determined by HPLC using the method described in Figure 7.2. The mobile phase used was the mixture of DDW 1 L, acetronitrile 900 ml, methanol 100 ml and orthophosphoric acid 1 ml. A flow rate of 1 ml/min was used. The HPLC column used was C18 Bondapak (150 mm x 3.9 mm) with a UV detector. All HPLC data were analysed by using a Powerchrom computer programme (version 2.0.7).

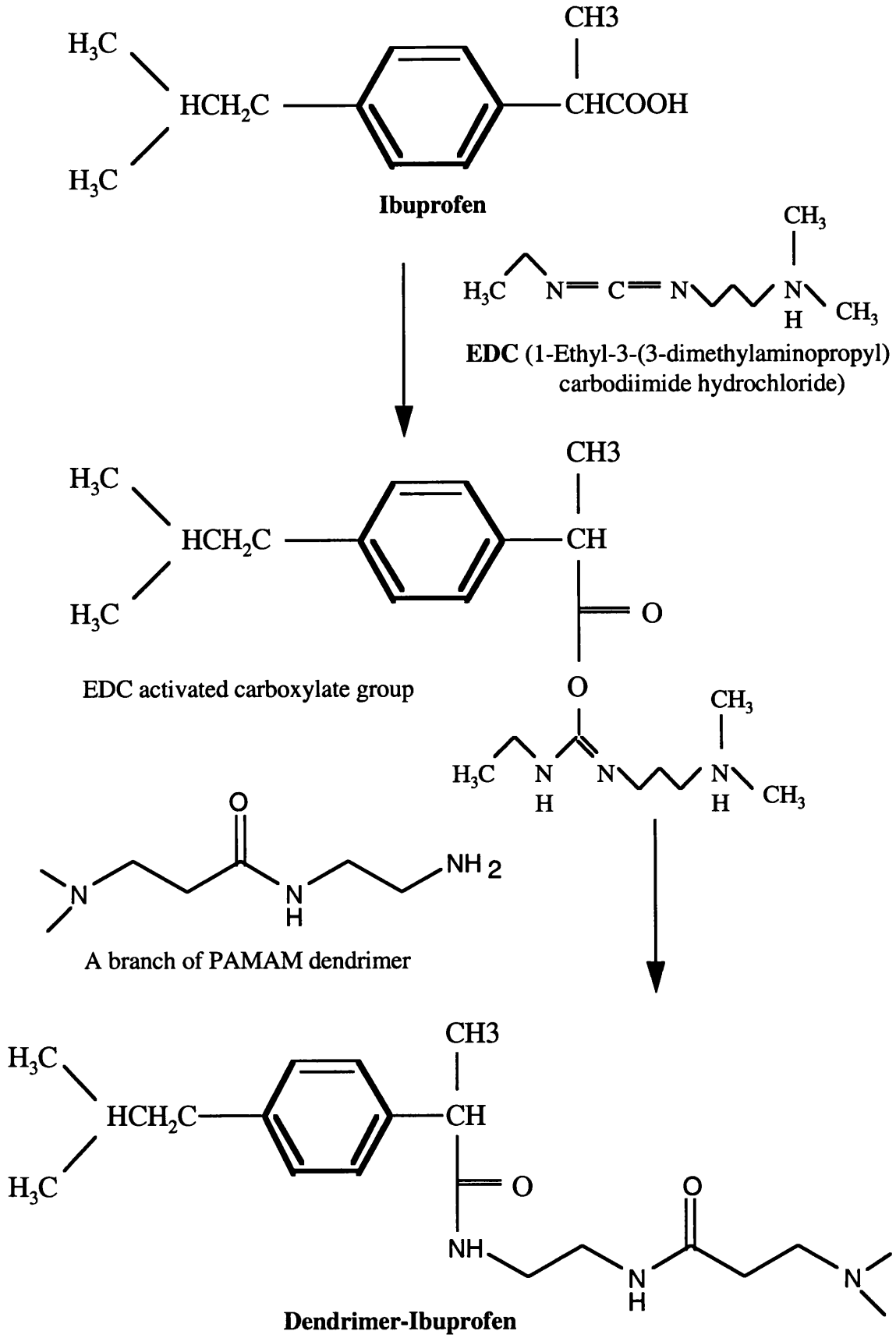
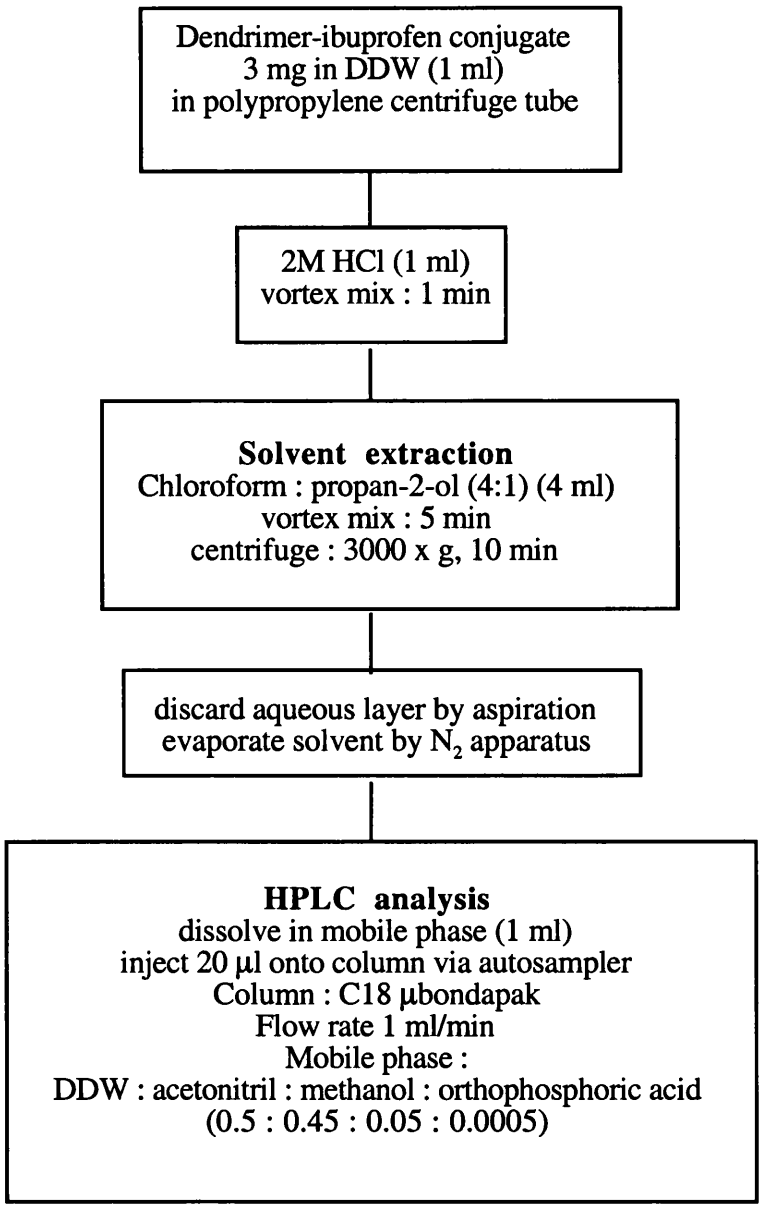


Figure 7.1. Dendrimer-ibuprofen synthesis

Determination of free ibuprofen



Note : The same extraction and HPLC analysis procedure was used for preparation of ibuprofen standard curve (10-50 µg/ml)

Figure 7.2. Method used for extraction and HPLC analysis of free ibuprofen in the dendrimer-ibuprofen conjugate

7.3. Results

Several batches of PAMAM dendrimer gen 3-ibuprofen were prepared using the EDC linker (Table 7.2). The GPC elution profile showed clearly the peaks corresponding to the conjugate and the free drug (Figure 7.3). A maximal loading of 13.4 wt % and a molar ratio of 1 dendrimer to 4.5 ibuprofen molecules was obtained after allowing 15 mole of drug to react with 1 mole of dendrimer (30.2 % efficiency) (Table 7.2). Increasing the amount of drug in the reaction mixture (25 : 1) resulted in precipitation of the conjugate.

^{1}H NMR profiles obtained for ibuprofen, PAMAM dendrimer (gen 3) and the dendrimer-ibuprofen conjugate are shown in Figure 7.4-7.6, respectively. The profile of conjugate (Figure 7.6) shows clearly the peaks of dendrimer appear between δ 2.3-3.6 ppm and the peaks of aromatic protons of ibuprofen appear at δ 7.0-7.1 ppm. The approximate molar ratio of dendrimer : drug calculated from the peak integral of the NMR profile was 1 : 4. Figure 7.7 shows the spectra obtained from HPLC analysis for ibuprofen standards. The amount of free ibuprofen in the conjugate was 0.08% of the total ibuprofen bound.

7.4. Discussion

In this preliminary study, water-soluble PAMAM dendrimer gen 3-ibuprofen conjugates were synthesised with a maximal drug loading of ~13% w/w. This is approximately a 1: 4 molar ratio of dendrimer : drug. Although PAMAM gen 3 contains 32 amine surface groups, it was difficult to get a higher drug loading. As ibuprofen is a hydrophobic molecule, the higher ratio of drug loading caused the reduction of the conjugate solubility resulting in precipitation of the conjugates. However, the ratio of drug loading in this study is considered high when compared with a PAMAM dendrimer gen 3.5-doxorubicin conjugate from previous studies (Malik 1999) which contains 2 molecules of dendrimer for 1 molecule of drug or 3 % w/w (PAMAM gen 3.5 has 64 carboxylate surface groups) using the same linker. There was very little of free ibuprofen in the product (~ 0.08%) as shown by HPLC analysis indicated the efficiency of the purification by the GPC.

It was intended to use this model system to investigate the ability of dendrimers to promote transfer of ibuprofen across the rat everted sac *in vitro* and also to increase oral absorption. Unfortunately due to lack of time it was not possible to undertake these studies here. However, the synthetic approach has shown the possibility to prepare well characterised conjugates and in future it is intended to replace the non-biodegradable linker with linkers able to mediate controlled release.

Table 7.2. Batches of dendrimer-ibuprofen

Ratio dendrimer : drug	Molar ratio obtained (dendrimer : drug)	Weight %	% Efficiency of coupling
1 : 10	1 : 3	9.0	29.3
1 : 15	1 : 4.5	13.4	30.2
1 : 20	1 : 3	9.0	15.7
1 : 25	*	*	*

* The values cannot be calculated due to the precipitation of the conjugate.

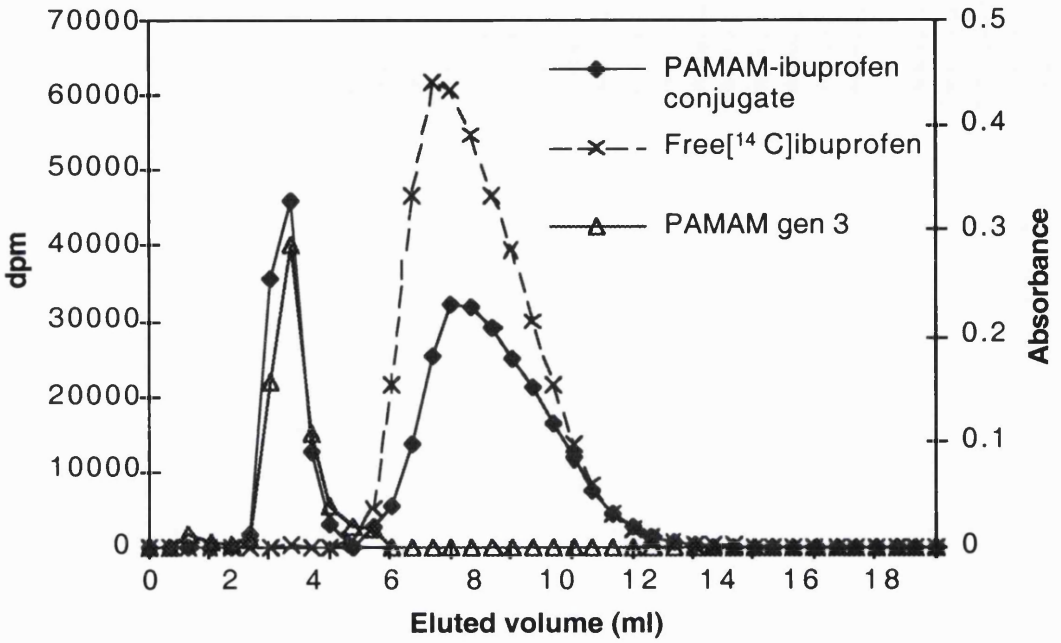


Figure 7.3. GPC elution profile of the reaction mixture and free ibuprofen. UV absorbance of PAMAM dendrimer is shown by the second Y-axis

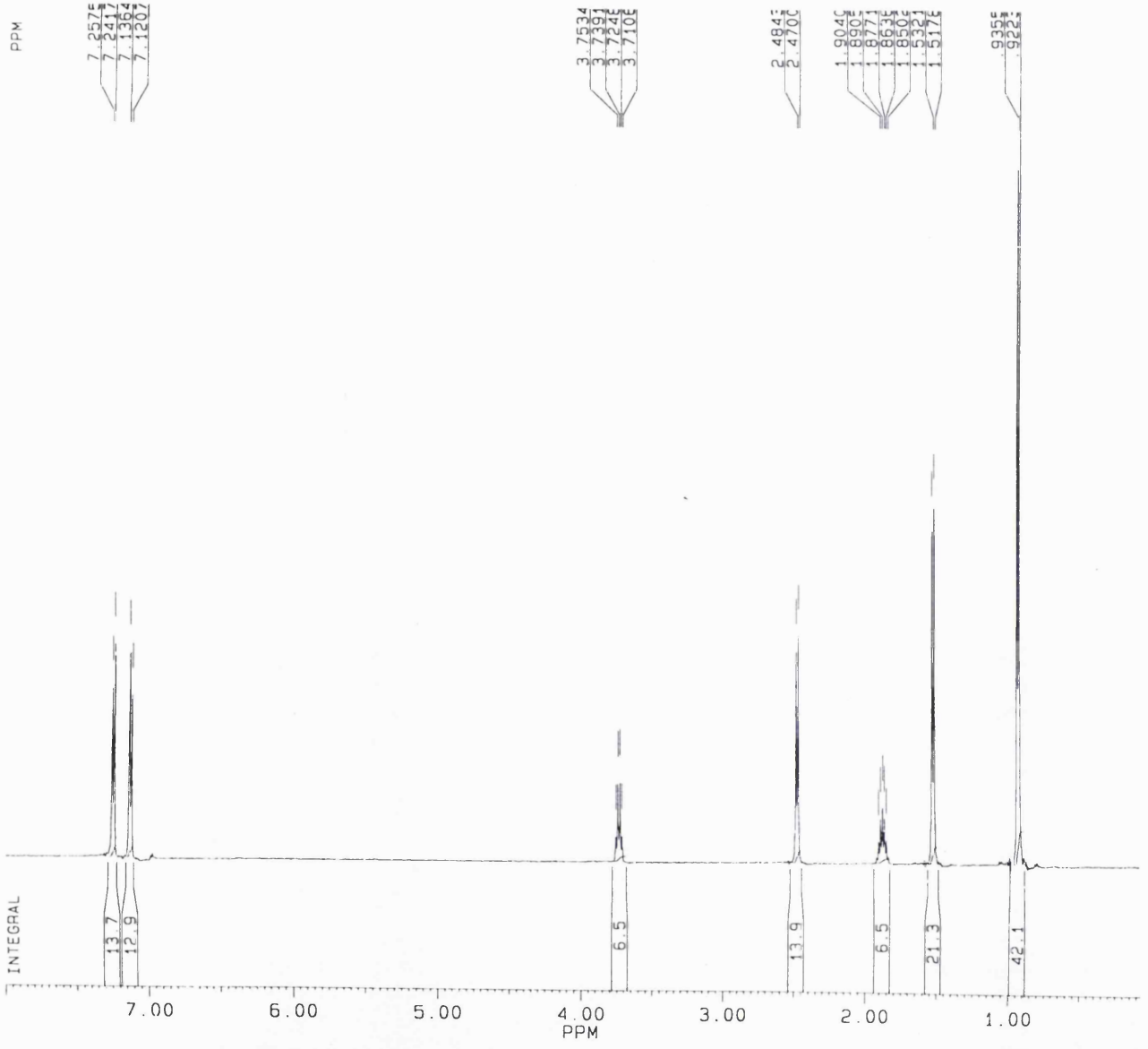


Figure 7.4. ^{1}H NMR profile for ibuprofen

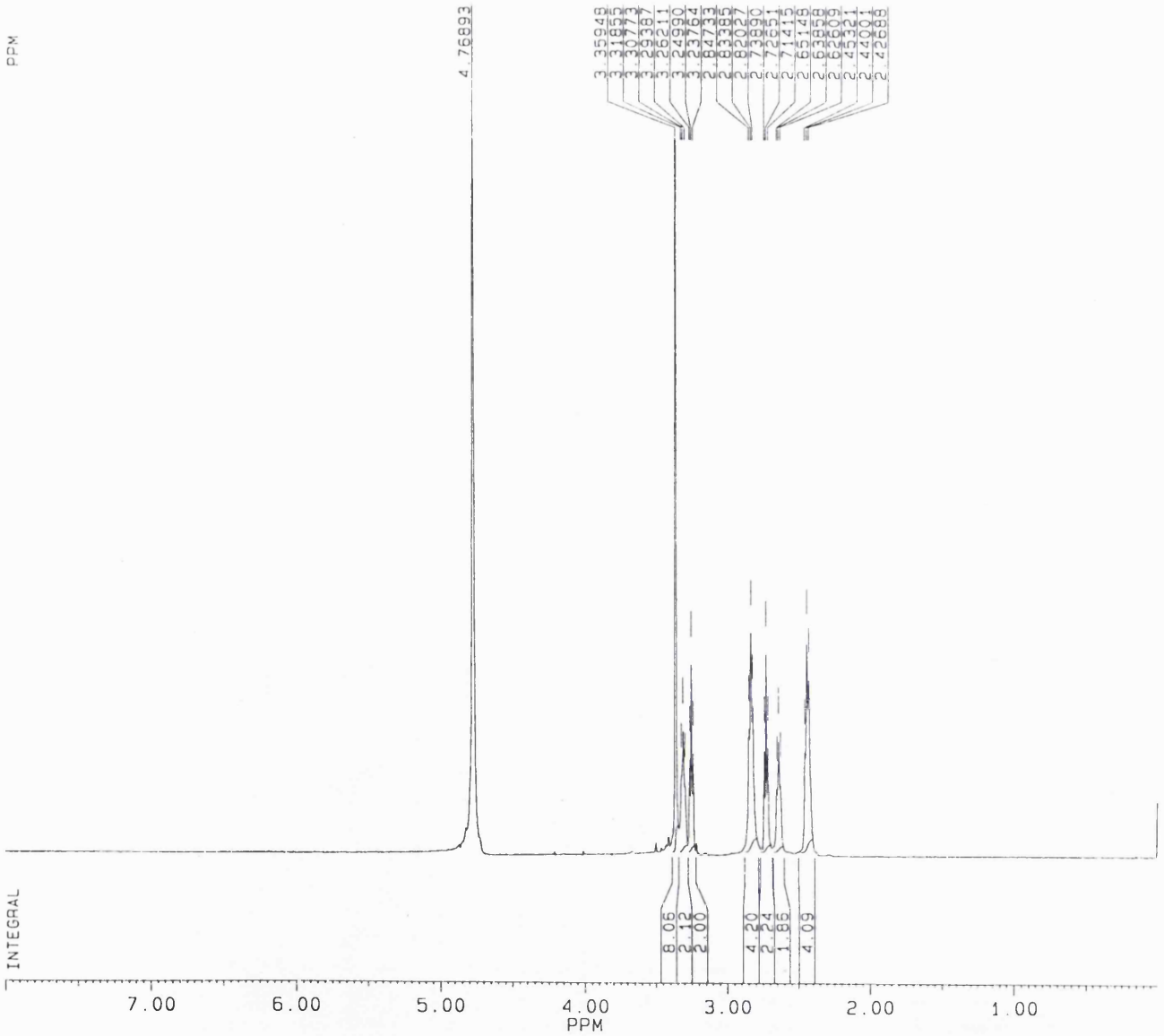


Figure 7.5. ^{1}H NMR profile for PAMAM gen 3

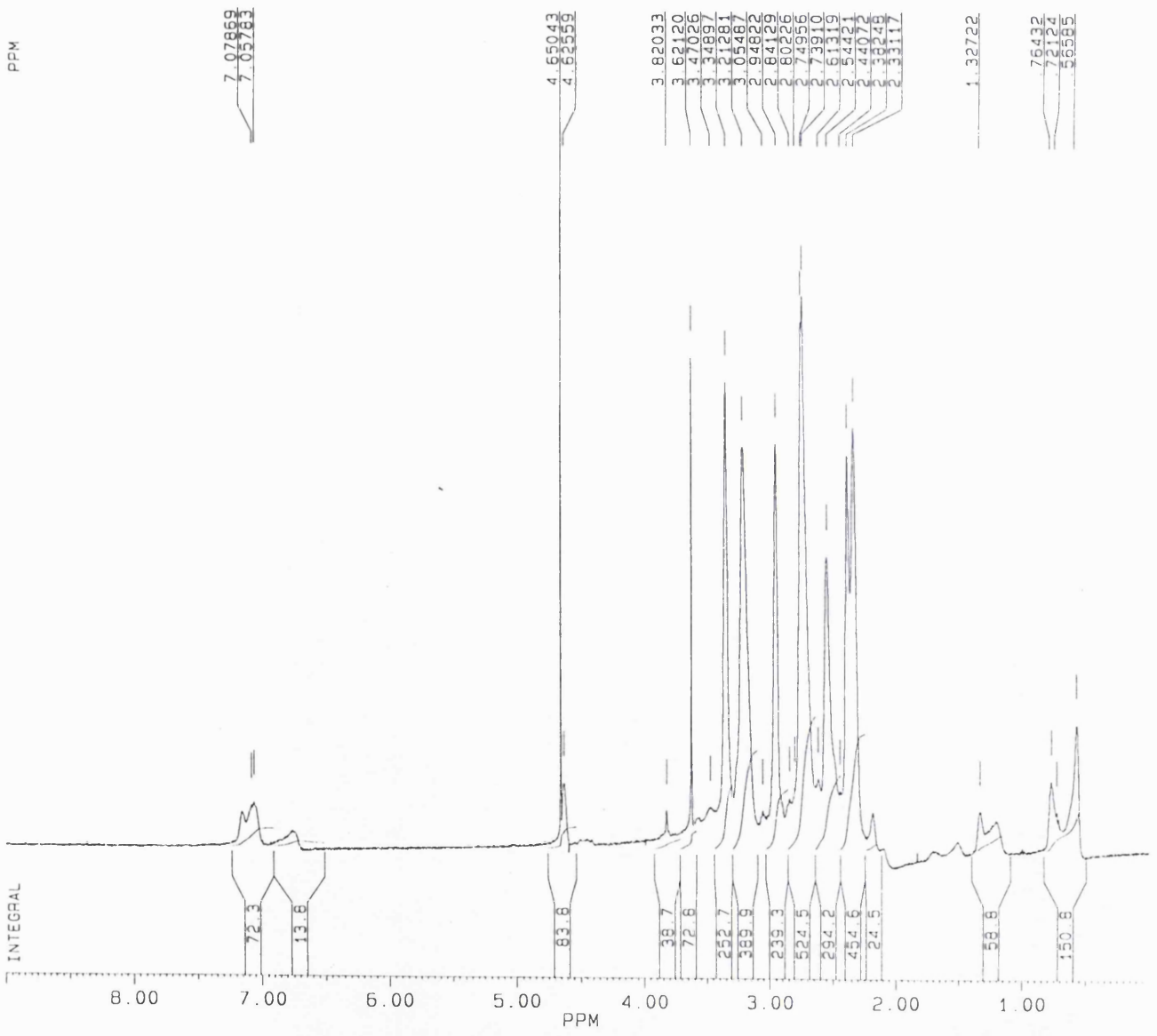
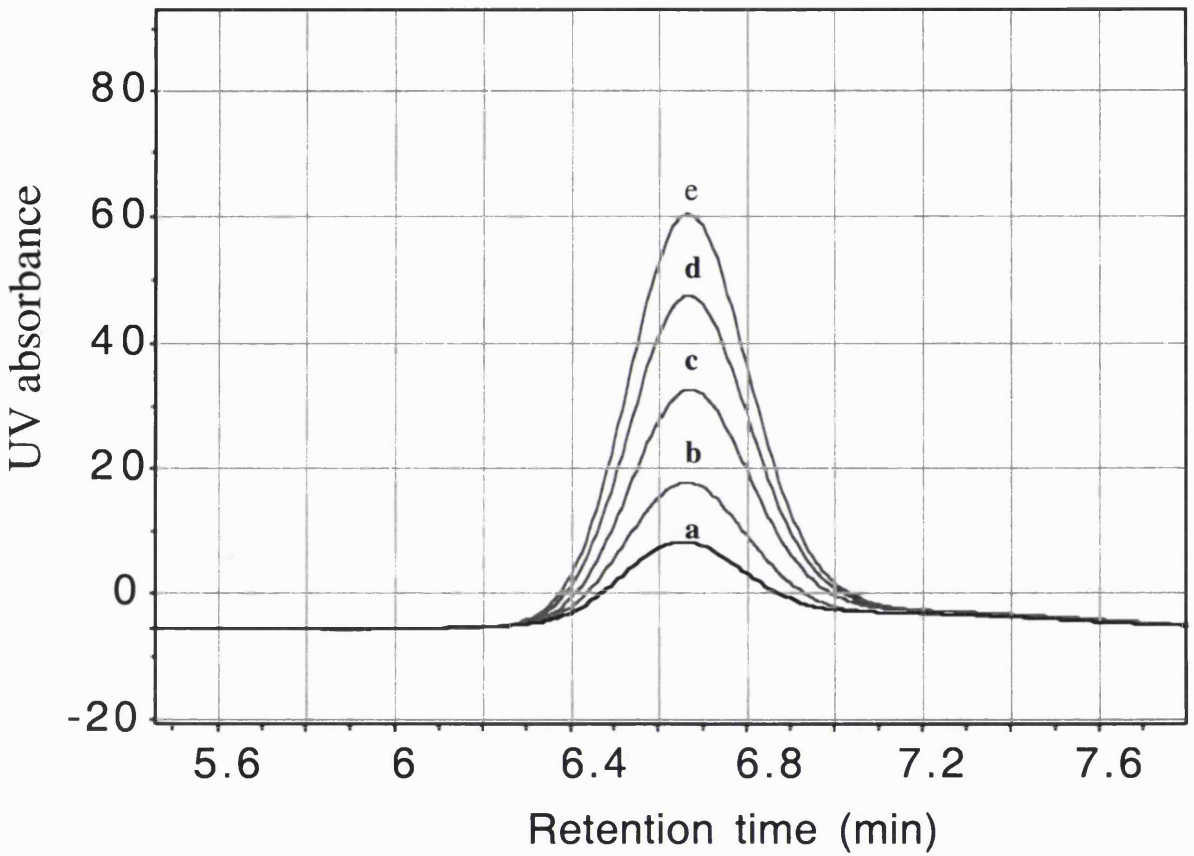


Figure 7.6. ^{1}H NMR profile for PAMAM-ibuprofen conjugate



Peak	Concentration (μg/ml)	Retention time	AUC
a	10	6.66	404.5
b	20	6.67	605.7
c	30	6.67	930.7
d	40	6.67	1247.4
e	50	6.67	1512.0

Figure 7.7. Spectra obtained from HPLC for ibuprofen standards

Chapter Eight

General Discussion

8.1. Dendrimers for drug delivery

Since the synthesis of the first dendrimers in the 1980s (Newkome et al 1985; Tomalia et al 1985; Hawker & Frechet 1990) these synthetic macromolecules have attracted much interest due to their unique architecture and special properties as introduced in Chapter 1. In 1996 at the beginning of this study, however, most studies involving dendrimers had been concerned with either the development of suitable synthetic protocols (to produce molecules with a well defined gen number), or were attemping to solve the problems associated with dendrimer isolation and characterisation. At that time there were very few reports describing the biological properties of dendrimers or their application in the drug delivery field.

Only recently have dendrimers begun to be evaluated as drug carriers (reviewed by Uhrich 1997, Bosman et al. 1999). A few studies describing their biocompatibility, biodegradation and pharmacokinetics *in vivo* have been undertaken (as reviewed in chapter 1), but there is still much to learn. The broad applications of dendrimers in drug delivery is summarized in Figure 8.1 although at the present time only the parenteral and oral routes of administration have been explored in any depth.

8.1.1. Dendrimer biocompatibility

Over the last three years several studies on the biocompatibility of dendrimers have demonstrated that dendrimer gen and surface functionality have considerable influence on their biological behaviour. Larger cationic dendrimers are toxic to cells and lytic towards RBC, whilst anionic dendrimers have been shown to be non toxic (Roberts et al 1996; Malik et al 1999). The data obtained in this study (Chapter 3) clearly showed that the number of cationic groups (NH_2) and the Mw (gen) of PVF copolymers and DAE dendrimers has an effect on their haemolytic activity and cytotoxicity. The observed toxicity of cationic DAE and cationic PAMAM dendrimers towards intestinal tissue also suggested that these cationic materials may be inherently toxic or irritating when given at high dose or repeatedly by oral administration. The experiments described in Chapter 3 also demonstrated the importance of early biocompatibility testing of novel materials as biocompatible candidates must be selected for further studies. It is important to note that other aspects of biocompatibility such as dendrimer immunogenicity and biodegradability still need further investigation before it is possible to confirm that dendrimers can be used in the real applications *in vivo*.

8.1.2. Dendrimers as carriers for parenteral delivery

Among the large number of different families of dendrimer synthesised, PAMAM dendrimers have been the most widely studied, largely because they are available in quantity, with a number of different surface groups and they are water soluble. The

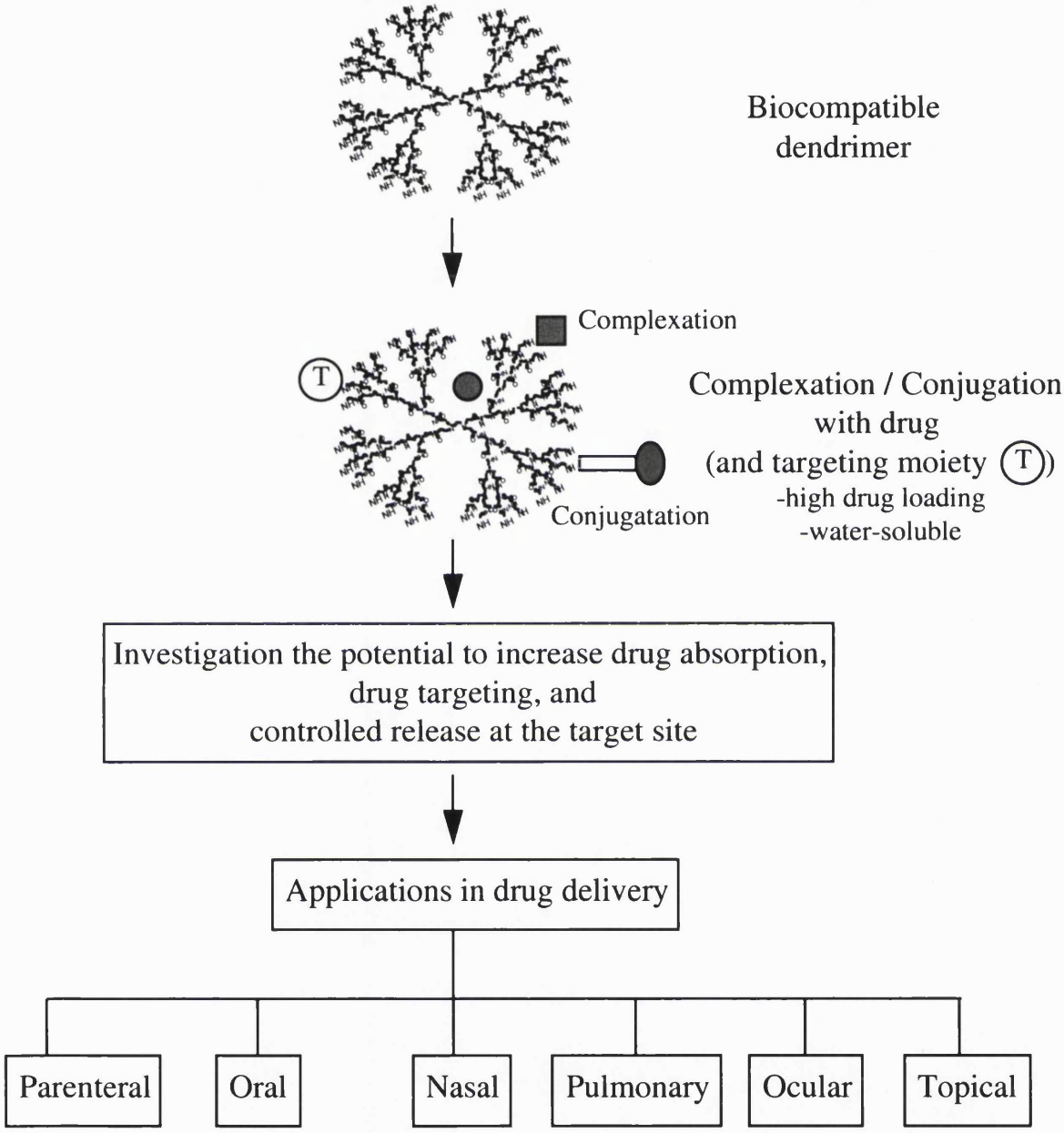


Figure 8.1. Potential application of dendrimers as drug delivery systems

studies that have examined the use of parenteral administration have mostly been investigating the use of dendrimers as carriers for anti-cancer agents. PAMAM dendrimer (gen 3.5)-cisplatin was the first dendrimer-anti-cancer based system (Malik et al. 1997). The dendrimer platinate was able to target solid tumour tissue B16F10 (melanoma) more effectively than cisplatin. The conjugate was also less toxic than cisplatin (Malik et al 1997). The selective accumulation of dendrimer-platinate observed in tumour tissue was due to the enhanced permeability and retention (EPR) effect. Using PAMAM dendrimer (gen 3) as a carrier for methotrexate, Khopard & Jain (1997) showed that the half-life and bioavailability of the drug was increased as a result of slow clearance of dendrimeric drug. Recently, the synthesis and *in vitro* release of 5-fluorouracil from cyclic core dendrimers were described (Zhou et al 1999). Dendrimers based on a core of 1, 4, 7, 10-tetraazacyclododecane were synthesised from gen 0.5 to 5.5. Full gens were also synthesised and characterised (gen 2-5). Parts of gen 4 and 5 amine surface were acetylated using acetic anhydride. The acetylated dendrimers were then reacted with 1-bromoacetyl-5-fluorouracil to produce dendrimer-5-fluorouracil conjugates. Hydrolysis of these conjugates in PBS caused release of free drug.

8.2. Dendrimers as vehicles for oral delivery

The work described in this thesis (Chapter 4) is the first study relating to the investigation of the potential of dendrimers as an oral drug delivery system. Interest in the use of dendrimers for oral delivery first arose as the dendrimer architecture provides a nano-particle structure of very small size-about the same size as natural macromolecules and as viruses (Tomalia 1990). Therefore, it was considered possible that these dendrimers might be taken into and across cells in a similar manner to viruses e.g. via the endocytic pathway. In addition, dendrimers were considered to have several advantages compared to linear polymers used as potential oral drug carriers (as reviewed in Chapter 1).

The possible role that dendrimers might play as macromolecular carriers for oral drug delivery are summarised in Figure 8.2. During the course of this study several of these potential uses were studied systematically. It was first considered important to determine whether dendrimers have potential to be orally absorbed. If dendrimers are absorbed they may be useful either 1) to deliver drugs to the enterocyte where a drug could be released in the lysosome or 2) as a carrier for systemic drug delivery. Alternatively, if a dendrimer is not orally absorbed, it may be possible to develop it as a carrier for drug delivery within the GI tract. Dendrimers could be designed to act as a bioadhesive drug delivery system to delay GI transit time, as a controlled release vehicle from which drugs might liberated by simple controlled release or by specific enzymes in particular region of GI tract. Alternatively, dendrimers might also be used to improve

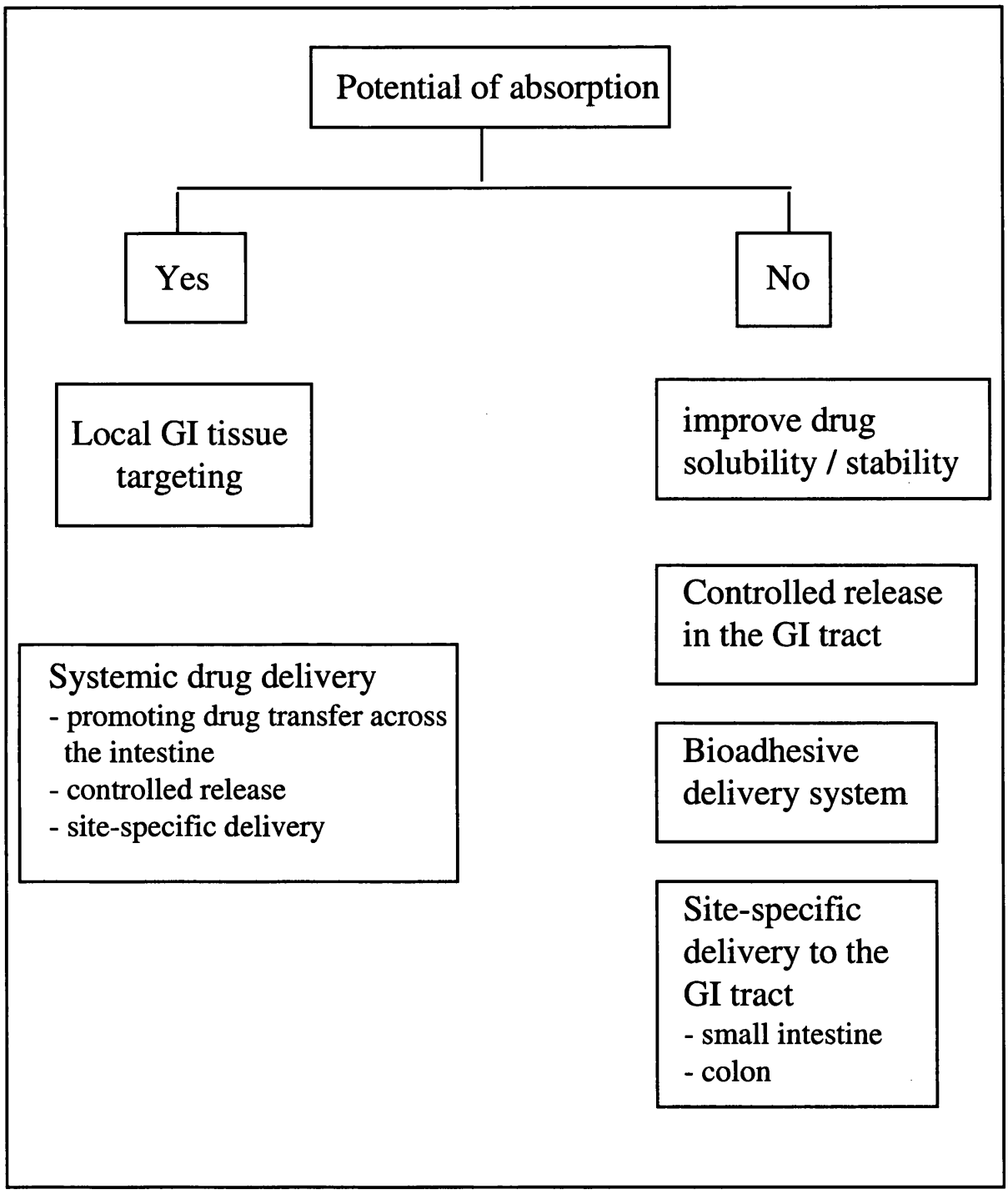


Figure 8.2. Possible roles of dendrimers as vehicles for oral delivery

solubility and oral bioavailability of drugs. These potential uses for dendrimers in the oral drug delivery systems of the future are discussed in more detail here.

8.2.1. Dendrimers: promotion of drug transfer across the GI epithelium

In chapter 4 it was shown that the size and surface functionality of dendrimers have considerable influence on the transepithelium transport mechanism. Anionic PAMAM dendrimers gen 2.5 and 3.5 showed particularly rapid serosal transfer which suggests they could be promising in promoting transfer of drug across the intestine. However, the GI transit time and body distribution of dendrimers seen after oral administration *in vivo* (Chapter 5) gave somewhat disappointing results without evidence of high systemic absorption of PAMAM dendrimers. The fact that 10-30% of the dose administered was not recovered leaves the interpretation of these data open.

Recently, the biodistribution of a lipidic peptide dendrimer was reported (Sakthivel 1999) and the properties of this dendrimer and its biodistribution compared to the PAMAM dendrimer used in this study are shown in Table 8.1. The lipidic dendrimer has smaller size than PAMAM gen 2.5-the smallest PAMAM dendrimer used in this study. It slightly showed higher levels of transfer (1.4-1.7% in urine) compared to the PAMAM dendrimers (0.1-0.2%). This was not surprising as previous studies have generally shown that hydrophobic molecules exhibit more absorption than hydrophilic molecules (Jani et al 1989; Pato et al 1994). In addition, the dose given for lipidic dendrimer (14 mg/kg) was nearly three time of the dose given for PAMAM dendrimer (5 mg/kg) in this experiment. The water solubility of PAMAM dendrimers may be more favourable in the term of pharmaceutical formulation compared to the lipidic dendrimer but this would depend on the compound to be delivered. In addition, the biocompatibility of PAMAM dendrimers has already proved that they are suitable for use parenterally (Roberts et al 1996; Malik et al 1999a), whereas the biocompatibility of lipidic dendrimers is not known. It still too early to make any firm conclusions regarding the possibility of using PAMAM dendrimers to promote oral absorption. Further experiments are needed to verify these observation made here, both *in vitro* and *in vivo* and the gamma camera imaging suggested in Chapter 5 will be very important.

Even if dendrimers do not have the ability to promote absorption, they still are promising for use in other ways as outlined in Figure 8.2. Here is a summary of their potential uses as an oral and other drug delivery systems which can be developed in the future. Some of them have been preliminary investigated in this thesis (Chapter 6 and 7).

Table 8.1. Comparison of physical and biological properties of PAMAM dendrimers with lipidic peptide dendrimer

Dendrimer properties	PAMAM dendrimers (gen 2.5, 3 and 3.5)	Lipidic peptide dendrimer (Sakthivel 1999)
Physical properties		
Mw	6,011 - 12, 419	6,300
Size (nm)	3.6 - 4.5	2.5
Solubility	water soluble	lipid soluble
Biological properties		
Biocompatibility	Anionics COO^- are safe, cationics NH_2 are toxic at high concentration	no information
Oral biodistribution		
- Radiolabelled used	^{125}I	^{14}C
- Dose (single)	5 mg/kg	14 mg/kg
- Radioactivity recovered in organs	0.1 - 1.2 % in blood at 1 and 5 h 1.4 - 7.8 % in urine at 24 h	3 % in blood, 1.2 % in liver and less than 1 % in spleen and kidney at 6 h
Biodegradable	non-biodegradable	biodegradable

8.3. Options for future development of dendrimers in drug delivery systems more generally

8.3.1. Dendrimers: improvement drug solubility and stability

The driving force for absorption of most drugs across GI membranes is the concentration of drug in solution. Poorly soluble compounds often show bioavailability problems. The currently used oral dosage forms for sparingly soluble compounds, often have a greater likelihood of local irritation in the GI tract i.e. anti-inflammatory drugs (Florence and Jani 1994). In the case of parenteral dosage forms, it is important that drug must be soluble and stable in the systemic circulation. Many strategies have been employed to overcome limited drug solubility including: use of cosolvents, solid solutions, micellar solubilization with surfactants, and increasing drug surface area through a reduction in particle size (Reviewed by Robinson 1993).

The results obtained in Chapter 6 demonstrate the ability of PAMAM dendrimers to solubilise model drugs. Electrostatic interaction and/or hydrogen bonding between drug and dendrimer is useful to solubilise drugs but it is clear that these interactions would not be stable enough to promote controlled release applications *in vivo*. Using apolar dendrimers with hydrophilic surfaces to solubilise drug by hydrophobic interaction will be another option (Frechet et al 1994). In this case the drug molecules are expected to be entrapped inside the core of dendrimer thus giving systems that would be more stable than seen in the case of electrostatic interaction. As the dendrimer surface would be expected to provide steric protection to drugs held in its interior, the stability of labile drugs may be improved using this method of entrapment. In addition, the ulcerative effects of drugs such as NSAIDS could be reduced by entrapment in the core. However, some dendrimers with a hydrophobic core have recently been shown to be haemolytic (Malik et al. 1999) it will be very important to evaluate the biocompatibility of such systems before conducting experiments *in vivo*.

8.3.2. Dendrimers: for controlled release

The incorporation of drug inside dendrimer molecule might allow controlled release depending on the size, shape and interaction between the drug and dendrimer. Very labile complexes will result in premature release of the drug and very stable complexes could result in retarded or incomplete release of the drug. In Chapter 6 it was shown that the simple complexation of piroxicam and indomethacin by PAMAM dendrimers was not able to mediate controlled drug release, very rapid liberation was seen. In contrast, Meijer's group have encapsulated guests into dendritic box (Jansen et al 1994). However, such strong entrapment is probably also unsuitable as the drug cannot be liberated from the carrier.

If a host dendrimer structure could be designed that could be opened by the local environment e.g. pH, enzymes, or solvent polarity then a remarkable controlled delivery system could be obtained. Conjugation of drugs to the dendrimer surface provides another mechanism to allow controlled release by biodegradable linkage. In Chapter 7, the preliminary conjugation of PAMAM dendrimer-ibuprofen via an amide bond has been studied, but this approach needs considerably more investigation if the correct linkers are to be introduced.

8.3.3. Dendrimers: for site-specific drug delivery to the GI tract

The aims of site-specific oral drug delivery to the GI tract can be divided into two categories: local treatment of inflammatory bowel diseases and improvement of oral absorption of drugs (Yoshino 1993). In future it will be interesting to design dendrimer molecules incorporating residues to promote site-specific drug delivery in the GI tract. Targeting motifs such as B12-intrinsic factor and transferrin could target to receptor sites on the brush border of the intestine (reviewed by Brayden & O'Mahony 1998). To prolong and/or intensify contact with the intestinal mucosa, specific bioadhesive e.g. lectins could be used.

8.3.4. Dendrimers as carriers for other routes of administration

Use of dendrimers as drug carriers for other routes of administration, (Figure 8.1) e.g. nasal, pulmonary, ocular and transdermal delivery system would be interesting to study in the future. Although each organ epithelium varies in cell type, thickness and function, the basic barrier role and transport mechanisms are the same as seen in the GI epithelium (Narawane & Lee 1994). In addition, administration of dendrimer-conjugates via these routes could avoid hepatic first-pass elimination, gut wall metabolism, and destruction in the GI tract. Understanding the nature of interactions between surface groups and cellular system by using atomic force microscopy (AFM) will be useful in the future in optimising these delivery systems (Black et al. 1999).

The potential use of dendrimers for drug delivery is still a new and exciting area. The experiments undertaken in this thesis are just the beginning of the development of dendrimers as an oral delivery system. It is expected that in the near future dendrimers will be specifically designed for oral delivery and the unique characteristics of these molecules suggest that they have the potential to become an important addition to the currently used oral drug delivery systems.

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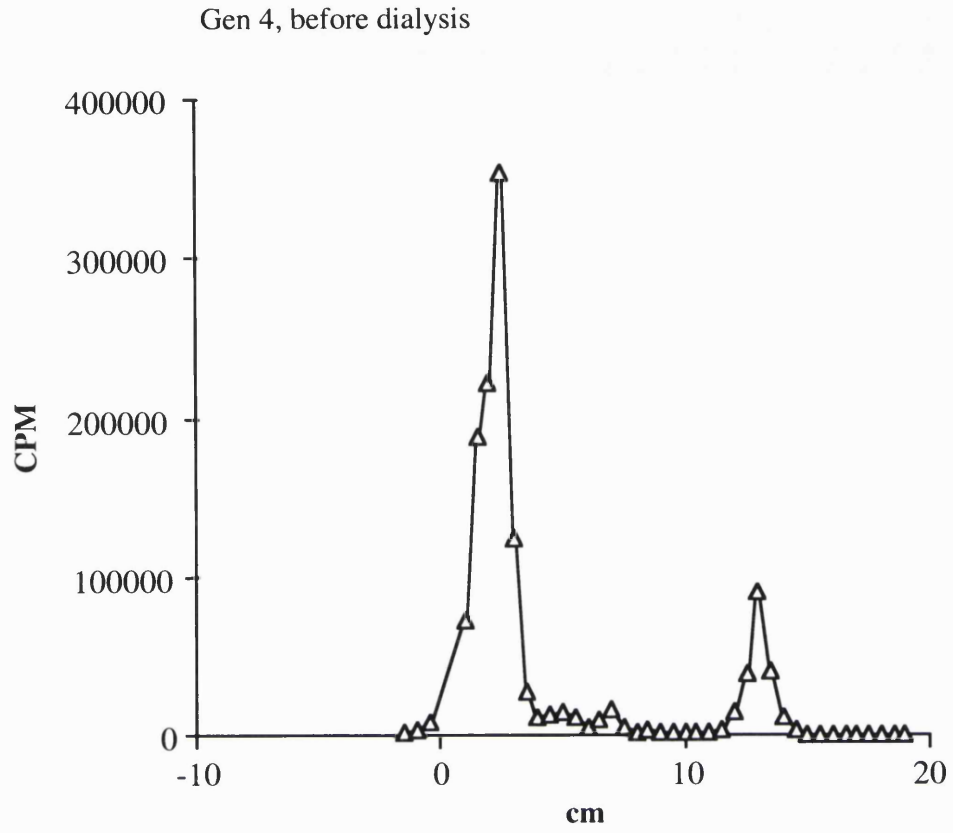
Appendix I



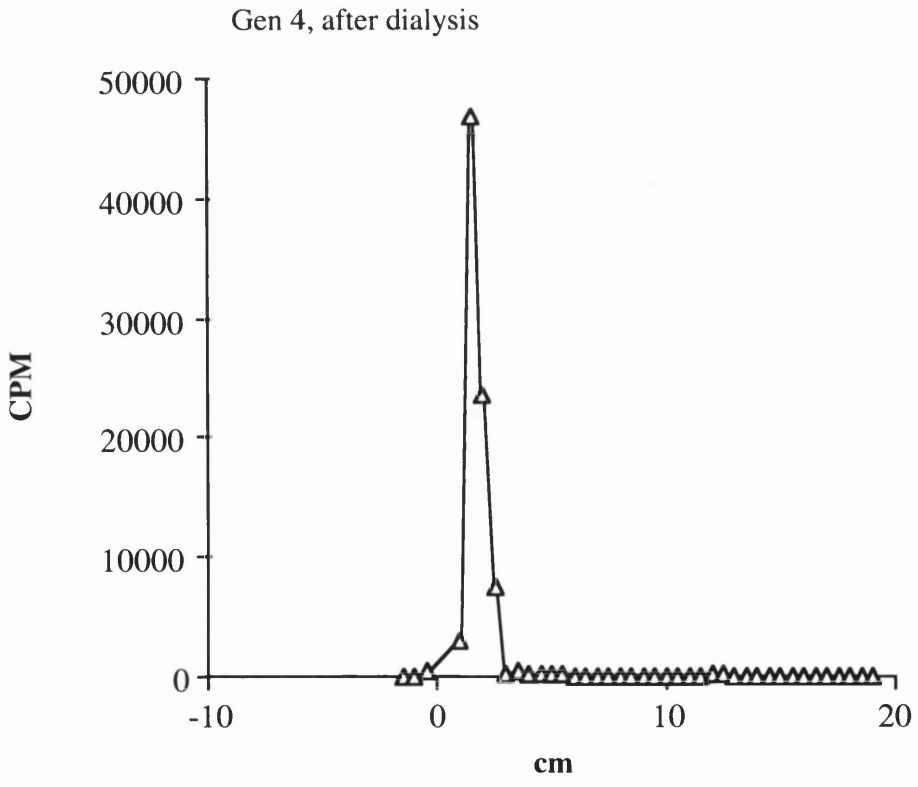
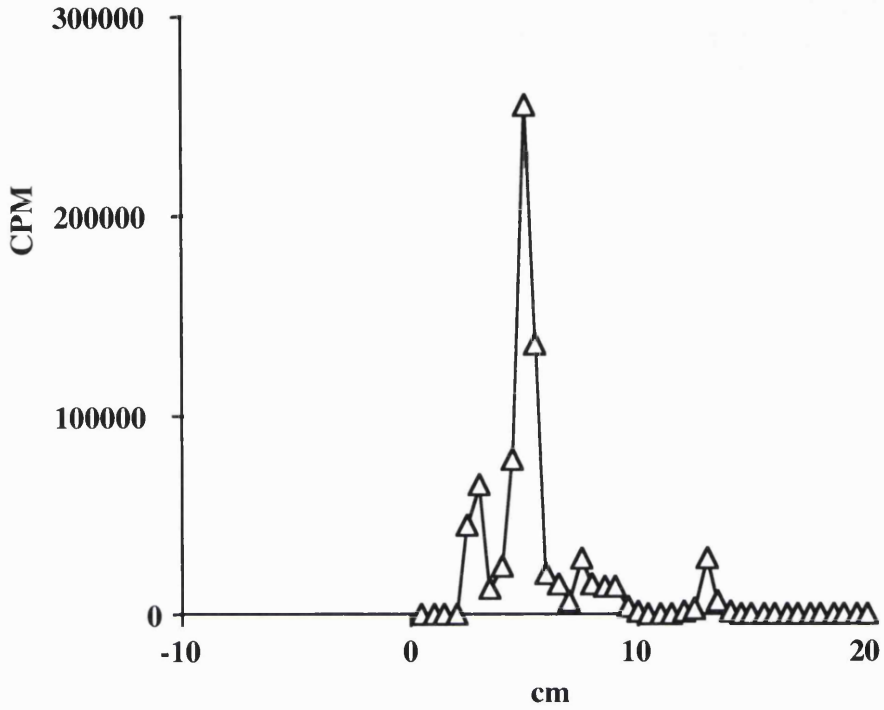


Figure 1. Paper electrophoresis analysis of cationic ^{125}I-labelled PAMAM dendrimer

Gen 5.5 before dialysis



Gen 5.5 after dialysis

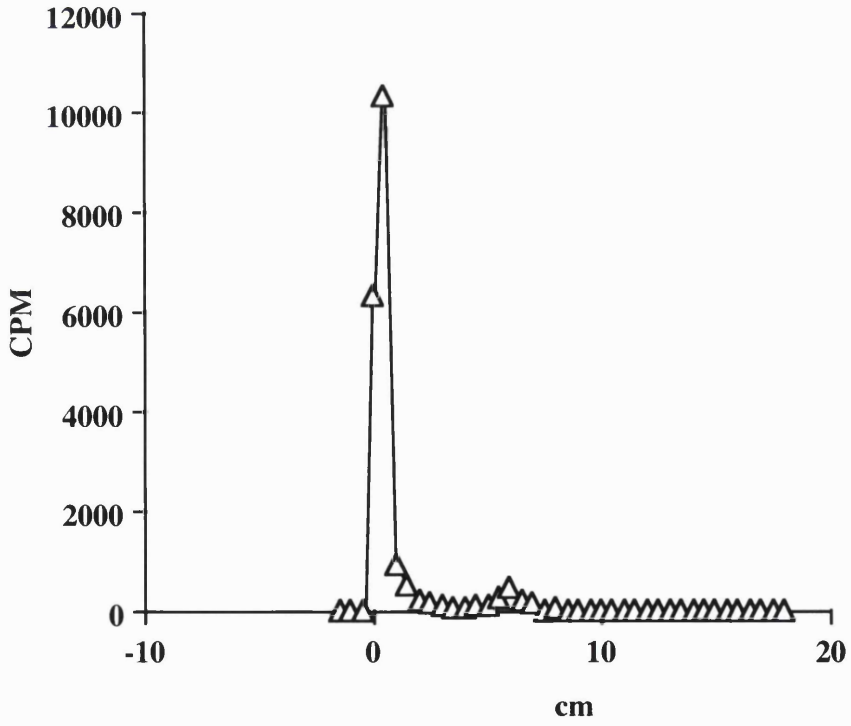


Figure 2. Paper electrophoresis analysis of anionic ^{125}I-labelled PAMAM dendrimer

Appendix II

List of Publications

Papers

1. Malik N, Wiwattanapatapee R, Klopsch R, Lorenz K, Frey H, Weener JW, Meijer EW, Paulus W and Duncan R, Dendrimers: Relationship between structure and biocompatibility *in vitro*, and preliminary studies on the biodistribution of ^{125}I-labelled PAMAM dendrimers *in vivo*, J. Control. Rel., in press (1999).

2. Wiwattanapatapee R, Carreno-Gomez B, Malik N and Duncan R, Anionic PAMAM dendrimers rapidly cross adult rat intestine *in vitro*: A potential oral delivery system, Pharm. Res., submitted (1999).

3. Wiwattanapatapee R, Jee R and Duncan R., Complexation of piroxicam and indomethacin with PAMAM dendrimers. Int. J. Pharm., in preparation (1999).

4. Wiwattanapatapee R and Duncan R, *In vivo* biodistribution of ^{125}I-labelled PAMAM dendrimers after oral administration to rats. J. Cont. Rel., in preparation (1999).

Abstracts

Oral presentation

These papers were selected for oral communication:

1. Wiwattanapatapee R, Paulus W and Duncan R, Preliminary biological evaluation of polyaminodendrimers, J. Pharm. Pharmacol., **49 (suppl)** , 28 (1997).

2. Wiwattanapatapee R, Carreno-Gomez B, Malik N and Duncan R, PAMAM dendrimers as potential oral drug delivery system : uptake to the rat intestine *in vitro*, J. Pharm. Pharmacol., **50 (suppl),** 99 (1998).

3. Wiwattanapatapee R, Jee RD and Duncan R, PAMAM dendrimers as a potential oral drug delivery system: dendrimer complexes with piroxicam, Proceedings of the International Symposium on Controlled Release of Bioactive Materials, Boston, USA, **26,** 145-146 (1999).

Posters

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Correction

Page 15 Line 3, delete 'oral'

Page 20 Section 1.1, Paragraph 2, Line 2, delete 'viscous'

Page 26 Line1, change 'about and' to 'about'

Page 32 Line 10, change 'is' to 'are'

Page 33 Paragraph 3, Line 6, change 'triton' to 'Triton'

Page 42 Line 4, change 'physiologic' to 'physiological'

Page 58 Paragraph 3, Line 6, delete 'to readhere'

Paragraph 3, Line 13, delete full stop

Page 62 Section 2.2.5, change 'an amine' to 'amines'

Page 67 Paragraph 2, Line 2 and Paragraph 3, Line 5, change 'sephadex' to Sephadex'

Page 74 line 9, During the incubation....., the polymer can interact with the cell membrane,.....disruption and haemoglobin release'

Page 83 Paragraph 3, Line 4, delete 'significantly'

Page 84 Figure 3.9.....B16F10 cells after incubation for 72h

Page 88 Figure 3.12..... serosal fluid after incubation for 30 min

Figure 3.13.....serosal fluid after incubation for 90 min

Page 96 Paragraph 3, change 'saltsolutions' to 'salt solutions'

Page 99 Section 4.3.1, change 'effiencies' to 'efficiencies'

Page 117 Line 2, change 'rat' to 'rats'

Paragraph 2, Line 5, change 'attached' to 'grafted'

Page 139 Paragraph 3, line 8, change 'radiolabeled' to 'radiolabelled'

Paragraph 3, line 9, change 'DTPA' to ' diethylenetriaminepentaacetic acid (DTPA)

Page 150 X-axis, change 'wavelenght' to 'wavelength'

Page 152 Figure 6.6.....dendrimer (1-5 mg/ml)