

STUDIES ON HYDROPHOBIC DENDRIMER NANOPARTICLES



A thesis presented by

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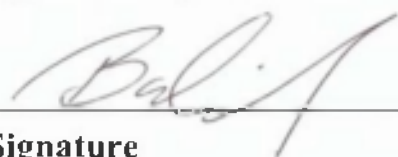
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This thesis describes research conducted in the School Of Pharmacy, University of London between October 2001 and September 2005 under the supervision of Prof. Alexander T. Florence. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

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Signature

18.01.06

Date

"The very essence of science is uncertainty"
A.K.

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Abstract

It has been over a decade since evidence was adduced for the enhanced translocation of nanoparticles through Peyer's patches of the gut associated lymphoid tissue. In the search for a biodegradable drug carrier with enhanced translocation from the gut, dendrimers of 2-7nm diameter and lipidic exterior were studied. These lipophilic dendrimers, synthesised using Fmoc solid phase peptide techniques, were characterised using mass spectrometry, NMR, HPLC, molecular modelling and monolayer formation. The dendrimers proved to be poorly soluble or insoluble in aqueous media and the opportunity was taken to formulate the dendrimers as nanoparticles by precipitation from solution in dichloromethane. The effect of concentration and surfactant on the diameter and stability of dendrimer-derived nanoparticles formed from two short homologous series of dendrimers - one 5th generation and one 6th generation series and with surface C₄, C₁₀ or C₁₂ groups - was investigated with photon correlation spectroscopy. Using pyrene (excitation 340nm) as a hydrophobic fluorescent probe, by measuring zeta potential and by studying the dendrimer at the air/water interface the packing of dendrimer-derived nanoparticles was assessed. Combined they made the basis for some calculations for the packing of dendrimer-derived nanoparticles. Calculations suggested a concentration and lipidic chain length dependent effect on packing density resulting in variable compact forms with hydrophobic interiors. The aggregates, derived from the 5th generation dendrimers with C₁₀ or C₁₂ surface lipidic chains, were studied in purified intestinal and stomach fluid, in which physical stability was assessed. Further aggregation of the dendrimer-aggregates occurs, more predominant in stomach fluid, because of an effect of pH and presence of salts, proteins and enzymes. Furthermore the dendrimer cytotoxicity was assessed *in vitro* using Caco-2 cells and red blood cells and made the foundation of the oral biodistribution study in animals using a radiolabelled dendrimer. The biodistribution was combined with histology to evaluate the uptake of the orally administered dendrimer. The study demonstrates that surface hydrophobicity, flexibility and concentration of the dendrimers, determines the size of the nanoparticles and their targeting to Peyer's patches and eventual translocation to the systemic circulation.

Abbreviations

APC:	Antigen Presenting Cells
CTB:	Cholera toxin B
DCM:	Dichloromethane
DMF:	Dimethylformamide
FAE:	Follicle-Associated Epithelium
Fmoc:	Fluorenylmethoxycarbonyl
GALT:	Gut-Associated Lymphoid Tissue
BALT:	Bronchus-Associated Lymphoid Tissue
GIT:	Gastrointestinal tract
H & E:	Hematoxylin and Eosin
LPPS	liquid phase peptide synthesis
MALT:	Mucosal-Associated Lymphoid Tissue
M-cells:	Membranous epithelial cells
MTT:	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide)
NALT:	Nasal-Associated Lymphoid Tissue
NLS:	Nuclear Localization Signal
PAMAM:	Poly(amidoamine)
PCS:	Photon correlation spectroscopy
PEG:	Polyethylene glycol
PLG:	Poly(D,L lactide-co-glycolide)
PP:	Peyer's patches
RBC:	Red blood cells
SPPS:	Solid phase peptide synthesis
TFA:	Trifluoroacetic acid

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Chapter one

Introduction

Dendrimers are three dimensional hyper-branched macromolecules (Tomalia 1990) with great potential as carriers because their surface properties can be modified to meet a variety of design criteria. Even though many structurally different dendrimers have been synthesised, the full potential of dendrimers has yet to be realised. Some dendrimers can self-assemble. If dendrimers are to be used as delivery systems, it is important to understand the nature of self-assembly and controlled aggregation with and without encapsulated drug. It has been well documented that physical characteristics including size and surface nature affect nano- and micro-particulate translocation *in vivo* (Florence 1997). Even though it has now been several decades since the first provided evidence of translocation of polystyrene nanoparticles ($< 1 \mu\text{m}$) through the Peyer's patches of the gut associated lymphoid tissue (GALT) (LeFevre *et al.* 1978a,b, 1980; Jani *et al.* 1989; LeFevre *et al.* 1989; Eldridge *et al.* 1990; Jani *et al.* 1990, 1992 a,b; Rolland 1993), it has created much debate and the evidence has been difficult for some to accept. It is now generally accepted that

nanoparticles ($< 1\ \mu\text{m}$) are taken up by the Peyer's patches (PP) of the gut associated lymphoid tissue (GALT) but the search for an optimal size and a biodegradable carrier which is translocated in sufficient amounts is still on going. In this thesis the properties, characterisation and ultimately the oral uptake of lipidic dendrimers aggregated into nanoparticles will be assessed.

1.1 General introduction

Nanoparticle uptake from the gut has been suggested to be important as an additional route of entry to the systemic circulation (Hussain *et al.* 2001). Potential applications include vaccine delivery, gene delivery and drug delivery, although the true potential of these applications depends on the progress in understanding particulate uptake from the gastrointestinal (GI) tract.

Various routes for mucosal delivery are being researched. The oral route of delivery is the most attractive and acceptable, but it is also the most challenging. In addition to oral delivery, intranasal delivery is also attractive whereas alternative routes, which might be successfully exploited in certain circumstances, include pulmonary inhalation, rectal and ocular delivery (O'Hagan 1998).

Nanoparticle/microspheres/nanocapsules can protect encapsulated labile materials against proteolytic enzymes, stomach acid (Norris *et al.* 1998) and nuclease degradation (Demaneche *et al.* 2001). Even surface adsorption onto nanoparticles has been shown to protect adsorbed molecules against degradation. Particulates may be taken up albeit in small quantities by the Peyer's patches of the gut associated lymphoid tissue to induce an immune response or reach the systemic circulation.

1.2 The mucosal system

The bulk of lymphoid tissue that is found in association with mucosal surfaces is called the mucosal-associated lymphoid tissue (MALT). The mucosal surface area is $\sim 400\text{m}^2$, and is the site of first encounter of immune cells with antigens entering via mucosal surfaces. Thus the lymphoid tissues are associated with surfaces lining the intestinal tract (gut-associated lymphoid tissue, or GALT), the respiratory tract (bronchus-associated lymphoid tissue, or BALT, which also includes the nasal-associated lymphoid tissue, or NALT) and the genitourinary tract (Roitt *et al.* 2000).

The epithelial surface covering the mucosal lymphoid tissue is named the follicle-associated epithelium (FAE). This epithelium contains membranous epithelial (M) cells that are specialized for endocytosis/transcytosis of antigens and microorganisms to the organized lymphoid tissue within the mucosa (Yeh *et al.* 1998). After entering into the MALT, the antigens are rapidly internalised and processed by antigen presenting cells (APC), such as subepithelial dendritic cells and macrophages, and presented to B and T cells located in the MALT. Exploiting these mechanisms, it has also been suggested, that colloidal carriers can be absorbed by way of gut associated lymphoid tissue (LeFevre *et al.* 1978a,b, 1980; Jani *et al.* 1989; LeFevre *et al.* 1989; Eldridge *et al.* 1990; Jani *et al.* 1990, 1992 a,b; Rolland 1993; Hillery *et al.* 1994; Sakthivel *et al.* 1999; Florence *et al.* 2000).

1.2.1 Gut-associated lymphoid tissue

More than 50% of the body's lymphoid tissues are found associated with the mucosal system, especially the GALT. Anatomically, the GALT consists of the Peyer's patches, the appendix, and the solitary lymph nodes in the gastrointestinal tract. The

Peyer's patches are found in the lower ileum. The intestinal epithelium overlying the Peyer's patches has been named follicle-associated epithelium, specialized to allow the transport of pathogens into the lymphoid tissue. This particular function is carried out by epithelial cells called M-cells (membranous epithelial cells), which contains deep invaginations of the basolateral plasma membrane which form pockets containing B and T lymphocytes, dendritic cells and macrophages (Figure 1.1). There are few defensin and lysozyme producing Paneth cells in the FAE (Giannasca *et al.* 1994) and low levels of membrane-associated digestive hydrolases (Bhalla and Owen, 1983). These characteristics tend to encourage local contact of intact antigens and pathogens with the FAE surface.

Once in the secondary lymphoid tissue, the lymphocytes move to other lymphoid organs via the blood and lymph (Figure 1.2).

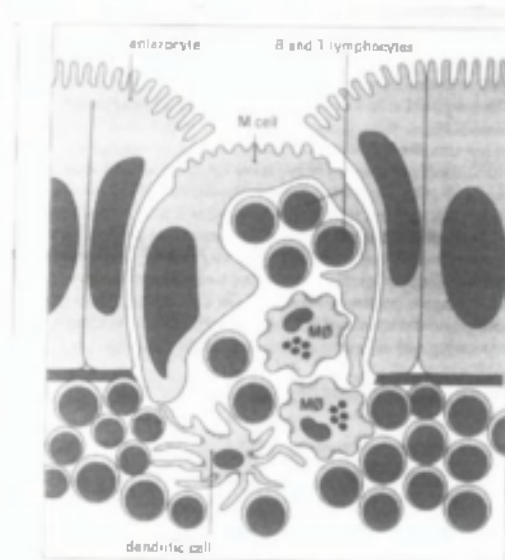


Figure 1.1. Endocytosed antigens are passed via intra-epithelial pockets into the subepithelial tissue (Roitt *et al.* 2000).

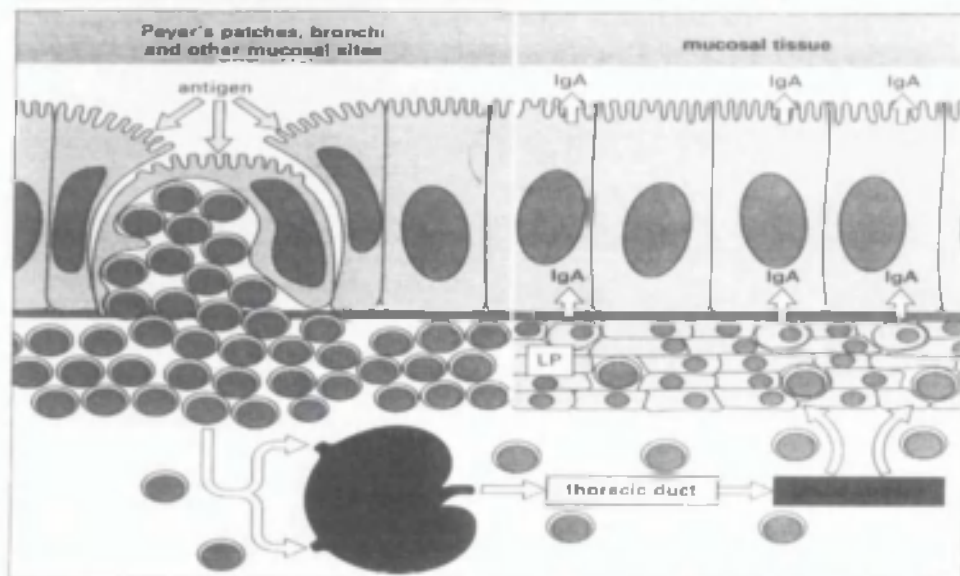


Figure 1.2. Lymphoid cells which are stimulated by antigen in Peyer's patches (or the bronchi or other mucosal sites) migrate via the regional lymph nodes and the thoracic duct into the blood stream. Therefore lymphocytes stimulated at one mucosal site can be distributed throughout the MALT system (Taken from Roitt et al. 2000).

1.3 Particulate uptake

The ability of intact microparticles and nanoparticles to be absorbed through the gut wall have been difficult to accept, even though the topic has a history of at least a century (Florence 1997). In a review in 1997, Florence suggested that the oral absorption of microparticles and nanoparticles was neither exceptional nor unusual, and today it is generally accepted that nano-particles below about 1 μm in diameter are taken up by the gut wall although in small amounts. In this section, particulate delivery after oral administration is reviewed, starting from the buccal cavity and through to the systemic circulation. Physical stability of colloidal carriers and methods to protect particulates from degradation in the buccal cavity, stomach and in the intestine, will be discussed. The interaction, movement, size and surface chemistry of particulates for targeted delivery to the M-cells of the Peyer's patches

will also be reviewed. Peyer's patches are areas of intestinal epithelial lining, containing less mucus on the apical surface while also having low level of hydrolase activity. These features provide a possible route of entry for particulate delivery systems for drug, gene and vaccine delivery.

1.3.1 Factors affecting particulate uptake in the gastrointestinal tract

Prior to transcytosis and translocation of particles in the intestine, several factors influence the movement, accessibility and interaction with the mucus (Table 1.1).

Table 1.1. Factors prior to transcytosis and translocation

- ◆ Physical stability of carriers in the gut, but also in the buccal cavity and the stomach
- ◆ Chemical stability of carrier and drug
- ◆ Transit time down the GI tract
- ◆ Residence time in regions of particle uptake
- ◆ Interaction with the gut content, e.g. adsorbing molecules
- ◆ Transport through mucus
- ◆ Adhesion to epithelial surfaces
- ◆ Stimulus for cellular uptake

Taken from Florence and Hussain (2001).

In order to formulate a particulate delivery system, that will successfully target M-cell Peyer's patches and internalise particles, it is important to study the factors listed

in Table 1.1. Particle stability in the gut depends on their interaction with the gut contents, which changes their physical nature, size and possibly surface characteristics, therefore affecting the interaction with the Peyer's patches and hence internalisation and migration through Peyer's patches.

Targeting of particulates has been under thorough investigation and will be discussed in section 1.3.4. Effective targeting is important, because it can result in increased adhesion to targeted tissue and hence affect factors such as transit time and residence time in the regions of uptake. However, effective targeting can only be achieved if there is significant stability of the carrier in the buccal cavity, stomach and the gut.

Another process that affects particulate uptake is the turn over or renewal time of the FAE in the intestinal epithelium. The total epithelial lining is normally replaced every 3-4 days (Smith *et al.* 1980; Bhalla and Owen 1982) in studies where repeated doses are given over a period of time, the natural renewal of the FAE must be considered of importance.

1.3.2 Stability of particles in the buccal cavity

It is important to note that after oral administration it is the buccal cavity that particulates first encounter, then the stomach and the gut. Even though the contact with the buccal cavity is brief, it may determine the subsequent fate of particles. Most oral uptake studies bypass the buccal cavity as particles are often administered directly into the stomach. The stability in the buccal cavity, however, will be of importance if and when particles are given to human volunteers for delivery studies, most notably because salivary proteins and oral bacteria binds to hydrophilic and

hydrophobic surfaces. Lassen *et al.* (1994) showed that materials rendered hydrophobic by methylsiloxane absorbed salivary constituents 25 times more than pegylated surfaces. The exact mode of interaction between particles or bacteria with saliva and mucus is still being studied. Enzymatic activity of peptidases, mainly aminopeptidases (Walker *et al.* 2002, Nielsen and Rassing 2000) but also carboxypeptidases (Douty *et al.* 1992), carboxypeptidase A (Nielsen and Rassing, 2000), dipeptidyl carboxypeptidases (Kashi and Lee, 1986), serine endopeptidase (Yamamoto *et al.* 1990) has been found. The enzymatic activity in the buccal cavity is low compared to the gastrointestinal tract, and therefore buccal administration could be of advantage for drugs and particles susceptible to degradation. Because particles only reside in the buccal cavity no more than 5 to 10 min after oral administration, the effect of enzymes and the mucosal lining is minimised. However, the stability in the buccal cavity is a prerequisite if oral delivery and uptake is to be successful. Whether it is local or intestinal delivery, it is an area still to be addressed.

1.3.3 Stability of particulates in the stomach.

The harsh environment in the stomach provides another big challenge for particulate delivery. The low pH combined with a high enzymatic activity, renders particles and pathogens susceptible to degradation. It is the body's first major defence and a difficult one to overcome. However, many dissolution studies have been conducted, and enteric coating of tablets with polymers provides one of the most common ways to protect against the low pH. An alternative method to improve pH barriers and hence improve oral bioavailability of poorly water soluble drugs is formation of pH sensitive polymeric micelles (Sant *et al.* 2005). Self assemblies formed from pH sensitive polymers composed of poly(ethylene glycol)-block-poly(alkyl acrylate-co-

methacrylic acid) (PEG-b-P(ALA-co-MAA), were nanoaggregates at pH values below 4.7. At pH values above 4.7, the polymeric micelles dissociated, explained by the ionisation of carboxylic groups present in the hydrophobic part of the copolymer chains. Using this system the assembly would remain intact at the low pH of the stomach and therefore prevent release of entrapped drug. As the polymeric micelles reach the small intestine, they would dissociate as an effect of the high pH (>5). This provides an interesting concept for oral particulate delivery and if adapted to other aggregated systems i.e. by incorporating structurally similar polymers or functional groups, could increase particles stability. The enzymatic degradation of these polymeric micelles was not studied. It, however, seems to be of importance as it could influence results.

Enzymatic degradation of particles and drugs in gastric fluid has been studied. Most commonly simulated gastric fluid containing pepsin (*USP Pharmacopoeia*). Venkatesan and Vyas (2000) stabilised liposomes made of egg phosphatidylcholine, cholesterol and phosphatidylethanolamine in simulated gastric fluid, by coating them with the polysaccharide o-palmitoylpullulan. Pepsin digests peptide bonds (–CO–NH–) but as the liposomes described, do not contain any peptide bonds, the obvious effect is therefore a pH effect. *In vivo* however, the gastric content which also contains bile salts renders liposomes susceptible to dissolution and degradation in the gastrointestinal tract (O'Hagan 1994), and therefore also affects particulate stability. Utilising systems containing bile salts or systems using purified gastric fluid (Singh *et al.* 2003) might provide more information and hence help improve particulate delivery systems susceptible for degradation.

Another interesting concept of protecting a delivery system is observed using hydrogels. Based on *n*-alkyl methacryloylamino esters of various chain lengths, acrylic acid, and acrylamide crosslinked with 4,4-di(methacryloylamino)azobenzene. The swelling degree of the hydrogel in buffered pH 7.4, was shown to be very low compared to the low pH of the stomach (Yin *et al.* 2002). It was shown that the degradation of the hydrogel, was related to the degree of swelling. As the hydrogel swells the entrapped drug will be released.

These pH sensitive systems are one way of overcoming stability problems in the stomach, and hence targeting the gastro-intestinal tract. If particulates can be controlled to behave in a similar way to pH sensitive systems, it may provide another interesting concept in stabilising particulates against degradation in the stomach. For particulates, besides the properties of the polymeric building block, stabilisation could be provided by altering the density of which the building block is packed together thus providing a resistance against degradation.

What is then the effect of particulates on M-cells after entering the gut? In order to understand the concept of targeting and particulate uptake, it is important to try to comprehend the nature of M-cells in different animal species and the factors influencing their differentiation.

1.3.4 Targeting particulates to M-cells in the gastrointestinal tract

The number, size and distribution of Peyer's patches varies among species (Griebel and Hein, 1996). In rodents, approximately 5-10 Peyer's patches are found fairly evenly distributed in the intestine (Abe *et al.* 1977). In rabbits, Peyer's patches are

approximately 1 cm in diameter and contain 40-50 follicles (Faulk *et al.* 1970). Therefore, evaluation and discussion of data should be done keeping in mind the species of animals used as models. Furthermore, the abundance of M-cells in the FAE varies in different mucosal tissue and species. In humans, the small intestinal M-cells represent ~5% or less of the FAE (Cuvelier *et al.* 1994), in rodents ~10% of FAE and in rabbits 50% of the FAE (Smith *et al.* 1980; Pappo *et al.* 1988). Because M-cells normally only represents subpopulation of FAE cells, and the fact that the cumulative surface area of M-cell apical membranes throughout the intestine is estimated to be less than 1/10000th of normal enterocytes, effective targeting is a prerequisite for delivery of microparticles to mucosal sites. However, the differentiation of M-cells has been shown to be induced by microbial or environmental stimuli (Savidge *et al.* 1996). The increase in the number of M-cells has been shown during neonatal development (Roy *et al.* 1987), and following incubation of Peyer's patches with *Salmonella spp.* (Savidge *et al.* 1991). Additionally, *in vivo* studies have shown that bone-marrow transplantation in severe combined immunodeficient syngeneic mice induced the formation of M-cells, hence demonstrating that M-cell genesis is immunoregulated (Kerneis *et al.* 1997; Sharma *et al.* 1998). It has also been shown that short time exposure (1-3h) of a non-intestinal bacterium induced alteration of the FAE, including a marked increase in the number of fully operational M-cells (Meynell *et al.* 1999; Borghesi *et al.* 1999). These studies show that factors stimulating the differentiation of M-cells need to be investigated, in order to fully understand and exploit M-cells for drug and particulate delivery.

Particulate interaction with M-cells is greatly influenced by the surface properties of cells, which exhibit species related variation, and the particles (Jepson *et al.* 1996;

Florence *et al.* 1997; Dibiase *et al.* 1997; O'Hagan 1996; Florence *et al.* 1995; Zho *et al.* 2002; Yeh *et al.* 1998). While it is clear that M-cells possess the capability to transcytose synthetic particulate systems, absorption is variable between different model systems and in many cases occurs at very low levels. There is also very often considerable variation in the extent of particle interaction with FAE between different animals, Peyer's patches, domes and even different regions of FAE overlying a single lymphoid follicle (Jepson *et al.* 1993, Beier *et al.* 1998, Smith *et al.* 1995, Clark *et al.* 2001). In the following section the different strategies utilised for targeting Peyer's patches is discussed. Factors such as surface charge, hydrophobicity, particle size and adsorption of targeting agents such as adhesions, invasins and lectins has been studied and has shown to influence targeting and particulate uptake across the intestinal epithelium.

1.3.5 Particulate uptake across the gut

Figure 1.3 represents three possible routes of particulate entry identified, 1) through M-cells, 2) through normal epithelial cells (enterocytes) and 3) by paracellular means (Florence 1997). The degree to which particles are taken up by each of these 3 different routes after oral administration of micro- and nanoparticles is not fully determined. The mechanism of transepithelial transport of propranolol-dendrimer, has been proposed to be mainly via an endocytotic transcellular route (D'Emanuele *et al.* 2004). Several factors have been shown to effect the extent of uptake across the gut (Table 1.2).

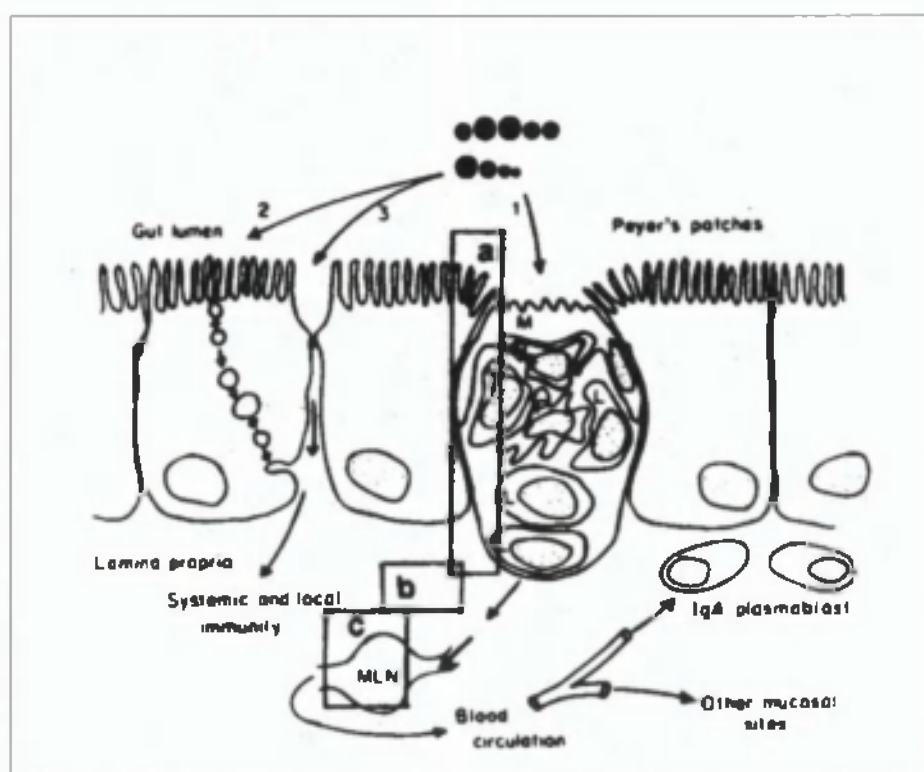


Figure 1.3. Representation of the three possible routes of entry of microparticulates into the lymphatic system or blood supply. Via : (1) M-cells of the Peyer's patches of the GALT; (2) Transcellular routes involving intestinal enterocytes; and (3) paracellular avenues through the tight junctions between cells (Florence 1997).

Table 1.2. Factors affecting the extent of uptake of microparticles across the gut.

Particle size (<5 μm and preferably in the submicron range)
Polymer composition
Particle hydrophobicity (adsorbed hydrophilic materials e.g. poloxamers reduce uptake)
Particle surface charge (lack of surface charge, charged particles less well absorbed)
Dose of particles
Administration vehicle
Animal species used for evaluation
Age of animals
Fed state of the animals
Use of targeting agents on particles (lectins and invasins increase adhesion and internalization)
Stability of the particle in the gut lumen

(Florence 1997)

Experiments have shown that particle absorption by the FAE may be modified by altering particle size (Eldridge *et al.* 1989, 1990; Tomizawa *et al.* 1993; Jani *et al.* 1989, 1990; Ebet *et al.* 1990; Damgè *et al.* 1996; Desai *et al.* 1996), hydrophobicity (Jepson *et al.* 1993a,b; Eldridge *et al.* 1990) and surface charge (Jani *et al.* 1989).

The main physical characteristics that affect particulate translocation are size and surface nature; the smaller the particle size, the greater the translocation. Charged surfaces reduce particle absorption, whereas increasing the hydrophobicity results in greater translocation. Particles with diameters from 3-10 μ m seemed to be sequestered within the Peyer's patches and do not migrate to the mesenteric lymph nodes, whereas 100nm particles diffuse throughout the submucosal layers (Eldridge *et al.* 1989). Experiments using rabbit Peyer's patch tissues and a range of cholera toxin B subunit (CTB) conjugates demonstrated that the glycocalyx acts as a size-dependent barrier which limits the interaction of particulates with the apical membranes of intestinal epithelial cells (Frey *et al.* 1996). Whereas FITC-conjugated CTB nanoparticles of 6.4nm in diameter, adhered equally to M-cells and enterocytes, the relatively thick glycocalyx overlying enterocytes prevented the interaction of colloidal gold- and latex microsphere-conjugated CTB (28.8nm and 1.13 μ m, respectively) with the apical membranes. The interaction of colloidal gold-conjugated CTB with the glycocalyx in the M cell apical membranes, was mediated by the thinner M cell glycocalyx, although this glycocalyx still inhibited the access of latex microspheres to the M cell apical membranes. These observations suggest, that the glycocalyx may restrict, in a size dependent manner, the access of reagents to receptors in the apical membranes of the intestinal epithelial cells. It has been suggested, that this problem can be avoided by restricting delivery vehicles to the nanometer size range or by selective targeting.

In the intestine, the kinetics of particle translocation depends on diffusion through mucus, contact with enterocytes or M-cells, cellular trafficking and post translocation events (Hoet *et al.* 2004). Szentkuti (1997) showed that cationic latex nanoparticles interacted with the negatively charged mucus, whereas carboxylated fluorescent latex nanoparticles were able to diffuse through. Furthermore, they showed that 14nm polystyrene nanoparticles penetrated through the mucus in 2 min while 415nm particles took 30 min. Smaller particles permeated the mucus faster than larger particles, suggesting that translocation begins as soon as contact with the target area is made and therefore depends on transit time through the intestine.

Many different particles have been studied for oral uptake e.g. biodegradable polymeric particle, polystyrene, dead bacterial cells etc, most extensively poly(D,L-lactide-co-glycolide)(PLG) polymer but also water-soluble biodegradable polymers such as chitosan, starch, dextran, alginate and dendrimer. All these particles have shown potential as mucosal drug or vaccine carriers. Also lipid particles have been used for oral delivery but, as mentioned previously, stability problems have limited the use due to dissolution by intestinal detergents, and to degradation by intestinal phospholipases (Chen 2000). Latex nanoparticles (Hussain and Florence 1998; Florence *et al.* 1995), liposomes (Chen *et al.* 1996) and microparticles (Jenkins *et al.* 1994) have also been studied.

Several studies have shown that attachment of bacterial and plant ligands, such as invasins (Hussain and Florence 1998) or tomato lectin (Lehr *et al.* 1992; Hussain *et al.* 1997; Wirth *et al.* 1998; Carreno-Gomez *et al.* 1999; Russell-Jones *et al.* 1999; Clark *et al.* 2000; Ertl *et al.* 2000) further enhances absorption. Many lectins have been studied and has shown great potential for targeted delivery. Furthermore, lectins

seem to enhance uptake of particulates at the targeted areas. However, most of these studies have been conducted targeting sugars from the intestine of rodents. Because of the specificity of the lectins, identification and studies of sugar moieties specific for the human intestine is a requirement if particulate uptake is to be successful in humans. Identification of sugars specific for the human intestine would restrict the use of lectins to those that would specifically target the human intestine (Roth-walter *et al.* 2005).

The extent of uptake so far has not been great. Many studies have been conducted with latex particles, so the need for biodegradable carriers or other alternative carrier systems, such as dendrimers, exist for the oral delivery of pharmacological active agents e.g. oral vaccines.

In the search for a better carrier which is more extensively taken up by the gut, compounds such as dendrimers have been synthesized in our laboratories (Sakthivel *et al.* 1998). Dendritic polymers have a promising future as drug delivery vehicles. The hope is that dendrimers will enhance efficacy by improving delivery issues such as targeting, stability and bioavailability.

This thesis is primarily concerned with the synthesis, characterisation and study of hydrophobic dendrimers based on the lipidic amino acid structures which have been the staple of our laboratory (Sakthivel *et al.* 1999; Al-Jamal *et al.* 2003, 2005; Ramaswamy 2003)

1.4 Dendrimers

All essential biological structures (e.g. cells) required for life have been based on the building blocks derived from controlled organic nanostructures. The first step involves molecular evolution from atoms to small molecules. These building blocks provide the base for more complex nanostructures and define the size dimensions that determines molecular level factors required for initiating and sustaining life. Figure 1.4 (Tomalia, 2005) shows a nanoscale comparison of poly(amidoamine) (PAMAM) dendrimers with a biological cell, proteins, DNA, lipidic bilayer, atoms etc. therefore constructing systematic nanoscale structures with exact controlled size, shape and surface nature seems essential (Tomalia *et al.* (2003).

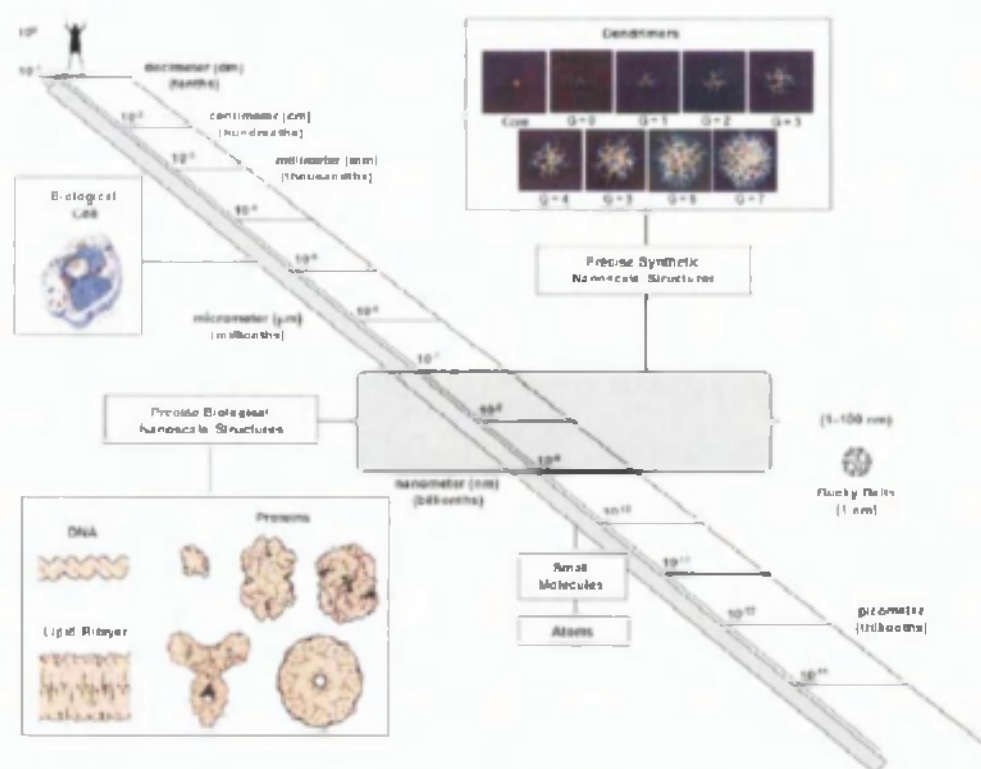


Figure 1.4. Nanoscale dimensional comparison of PAMAM dendrimers (generation 0-7) with a biological cell, proteins, DNA, lipid bilayer, bucky balls, small molecules and atoms (adapted from Tomalia, 2005)

Dendrimers are currently being developed by many laboratories as new particulate delivery agents. The optimal synthetic delivery systems are ones that have:

- Protection from enzymes
- Effective transport to the cells
- Avoidance of sequestration by the endosomal/lysosomal compartments
- Drug release at the required site
- The ability to target specific cell types

Dendrimers are highly branched and reactive three-dimensional macromolecules, with all bonds emanating from a central core (Figure 1.5).

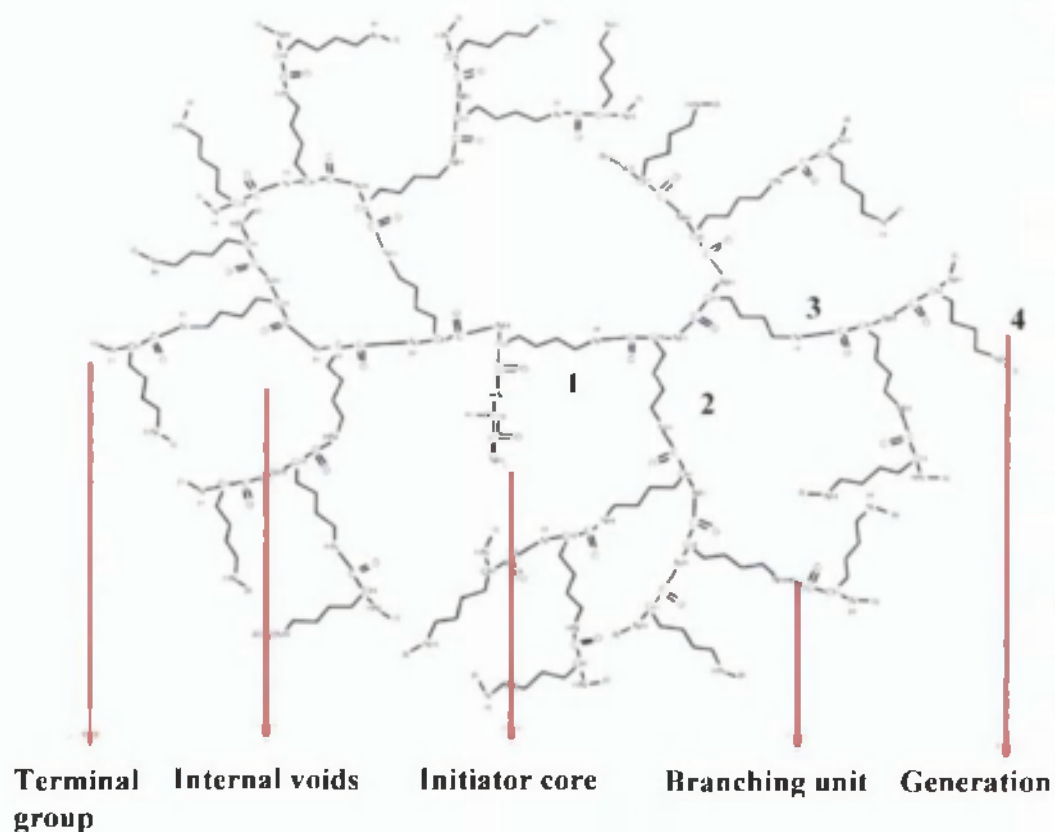


Figure 1.5. Showing the structure of a 4th generation lysine based dendrimer, with a glycine initiator core (Modified from Al-Jamal et al. 2005).

Since their introduction in the mid-1980s, this novel class of polymeric materials has attracted considerable attention because of their unique structure and properties. Compared with traditional linear polymers, dendrimers have much more accurately controlled structures, with a globular shape, a single molecular weight rather than a distribution of molecular weights, monodisperse size and a large number of controllable 'peripheral' functionalities (Tomalia *et al.* 1990). Many different types of dendrimers have been synthesised as shown in 1.3.

Table 1.3. Different types of dendrimers

PAMAM (Polyamidoamine dendrimers) (Tomalia <i>et al.</i> 1985)
<i>Denkewalter</i> (Polyamide dendrimers) (Denkewalter <i>et al.</i> 1981) including
<i>Polylysine-based dendrimers</i> (Sakthivel <i>et al.</i> 1998)
<i>Polyethyleneimine</i> (Tomalia <i>et al.</i> 1990)
<i>Hydrocarbon</i> (Hart <i>et al.</i> 1986)
<i>Polyether</i> (Padias <i>et al.</i> 1987)
<i>Polypropyleneimine</i> (Jansen <i>et al.</i> 1994)
<i>Dendritic acryl</i> (Hall <i>et al.</i> 1987)
<i>Polyamidoalcohol</i> (Newkome <i>et al.</i> 1985)

If these dendrimers are to be used successfully pharmaceutically, the dendrimers must be biodegradable. Unfortunately most of the interesting dendrimer structures created by the chemists are not. Therefore efforts have been made to generate dendrimers using naturally occurring reactive species like amino acids (Sakthivel *et al.* 1999).

Dendrimers can be used as potential drug delivery agents in at least two different ways:

- 1) Drug molecules can be physically entrapped inside the dendritic structure. There are three main strategies for encapsulation i) physically entrapment in the internal 'cavity' of the dendrimers, ii) multiple noncovalent chemical interactions, such as hydrogen bonding between guest molecule and the dendritic structure and iii) hydrophobic interactions.
- 2) Drug molecules can be covalently attached onto the surface or other functionalities to afford dendrimer-drug conjugates. Various molecules have been conjugated such as antibodies, sugars, platinates, folic acid and PEG (Liu *et al.* 1999, D'Emanuele *et al.* 2004).

Overall, these simple early designs have demonstrated that components can be varied to optimize capacity, solubility and rate of release as well as other physical and pharmacological properties. By carefully selecting the surface chemistry of dendrimers, it is possible to construct dendrimer-drug conjugates and provide the conjugates with a targeting feature by incorporating targeting moieties (Liu *et al.* 1999).

At the Centre of Drug delivery Research (CDDR) our group has focused on i) the presence and/or number of amino groups (Purohit *et al.* 2001), ii) the position of any lipid chain (Ramaswamy *et al.* 2003), iii) dendrimer drug interaction (heparin, penicillin) (Al-Jamal *et al.* 2003), iv) DNA interaction (Ramaswamy *et al.* 2003) v) self-assembly of dendrimers (Sakthivel *et al.* 1998) vi) movement of dendrimers inside cells (Ruenraroengsak *et al.* 2005). Dendrons (partial dendrimers) containing a nuclear localization signal (NLS) sequence have been studied in vitro (Toth *et al.*

1999) for gene delivery and as a function of the charge ratio of DNA (Shah *et al.* 2000). Furthermore the oral uptake and translocation of hydrophobic dendrimers has been studied (Sakthivel *et al.* 1999; Florence *et al.* 2000). The assembly of hydrophobic dendrimers into controlled aggregates has been studied and is described in this thesis.

Assembly of dendrimers into various structures has recently been the subject to intensive study. Various ways of interaction and structural formations are described in following section.

1.4.1 Dendrimer self-assembly

Self-assembly is a very interesting concept. The possibility of designing dendritic building blocks that can assemble into an array of different structures provides a tool that can be used in wide range of technologies, from material science to molecular biology (Whitesides and Grzybowski 2002).

Self-assembly relies on non-covalent interactions such as electrostatic interactions, hydrogen bonds, van der Waals' forces and solvophobic effects (Beer *et al.* 1999; Steed and Atwood, 2000). These forces provide the basis for controlling self-assembly with relative little synthetic input, which is a comparatively easy and therefore an attractive option when compared with the many synthetic steps needed for the synthesis of the similar structures. The synthesis of dendritic structures therefore provides the basis for assembly into more multifaceted structures (Figure 1.6) (Smith *et al.* 2005)

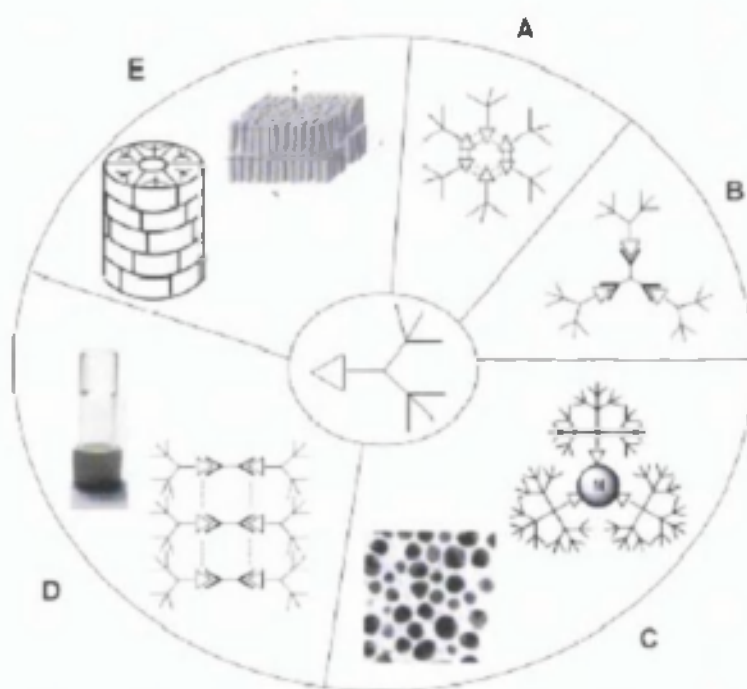


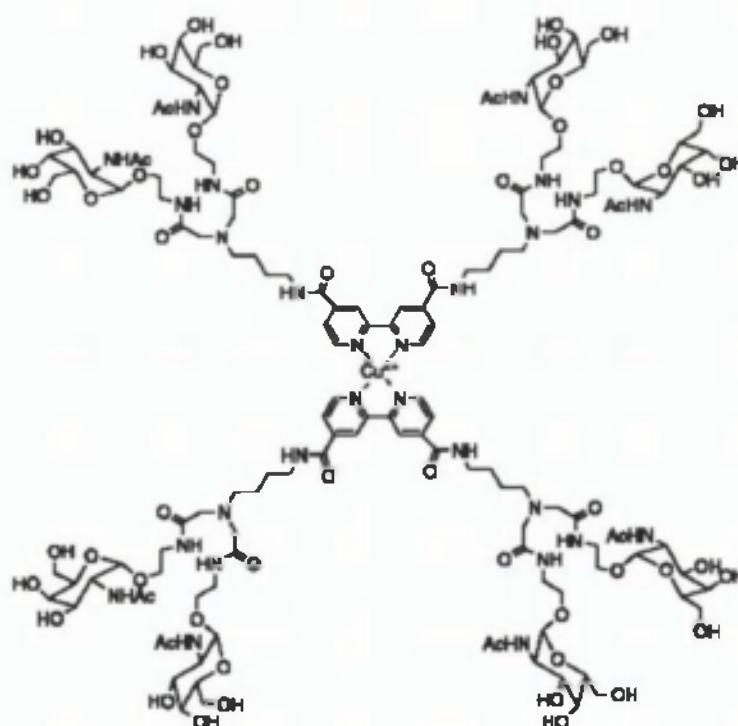
Figure 1.6. Schematic illustration of the self-assembly of dendritic building blocks. (A) Untemplated assembly of dendrons. (B) Templated assembly of dendrons. (C) Nanoparticles with assembled dendritic surface groups. (D) Fibrous, gel-phased assemblies of dendritic molecules. (E) Liquid crystalline assemblies of dendritic molecules. (from Smith et al. 2005).

As shown in Figure 1.6, assembly of dendritic structures can form a multitude of different structures. The assembly of dendritic structures can be defined in 3 different groups:

1. Self-assembly of well defined supramolecular dendrimers (A, B).
2. Self-assembly of dendritic matter into nanoscale structures, which exist as statistical distributions (C).
3. Self-assembly of dendritic matter which can extend into macroscopic structures (D, E).

- These will be described in more detail in the following section.

Self-assembly of dendritic matter into well defined supramolecular dendrimers, has mainly been achieved using, i) hydrogen bonding as means of interaction between dendritic structures (Zimmerman *et al.* 1996; Gillies *et al.* 2004), ii) organic molecules as template (Wang *et al.* 1997), iii) metal ions as a template (Newkome *et al.* 1997; 2003). Recently Roy *et al.* (2003) used dendrons containing a bipyridine core with surface saccharide functionalities. These were assembled around copper (II) ions see Figure 1.7 below. The lectin binding activity of the saccharide was investigated and showed greater activity than single dendrons. This underlines the potential of self-assembly to enhance biological potency.



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1.4.1.2 Self-assembly of dendritic matter into nanoscale structures, which exist as statistical distributions (2)

Such assembly has recently been reported by Tomalia *et al.* (2000) and Uppuluri *et al.* (2000) who formed *core-shell tecto(dendrimers)* which were formed using a self-assembly step. The PAMAM dendrimers used for the study contained carboxylate and ammonium functionality, which had a negative (3rd generation) and positive (5th generation) surface charge, respectively. By utilising an excess of third generation anionic dendrimer, in the presence of LiCl in water, a controlled core-shell assembly can be generated (Figure 1.8). These dendrimers could be covalently cross-linked together by addition of 1-(3-dimethylaminopropyl)3-ethylcarbodiimide hydrochloride. The formation of nanoclusters from the 7th and 5th generation PAMAM dendrimers was investigated by Betley *et al.* 2002, and again showed that it was possible to form controlled assemblies. The problems faced when assembling these dendrimers, is the additional formation of imperfect structures and aggregates.

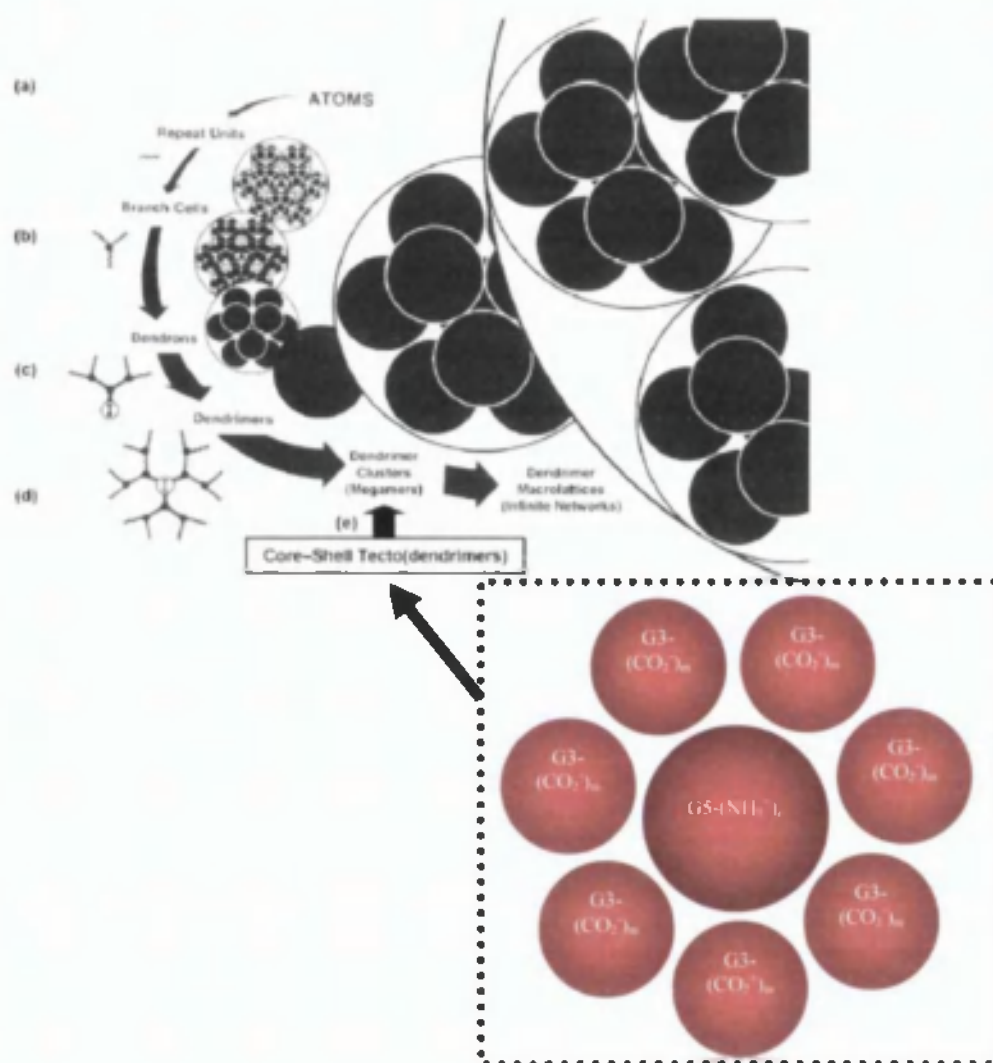


Figure 1.8. Hierarchy of construction components: (a) monomers, (b) branch cells, (c) dendrons, (d) dendrimer and (e) Core-shell tecto(dendrimer) assembled as a consequence of controlled electrostatic interactions (modified from Tomalia *et al.* 2000 and 2005).

The assembly of dendritic matter is not only limited to spherical structures. Kim *et al.* (2003) assembled cylindrical dendritic porphyrins to form hollow nanotubular structures. Sakthivel *et al.* (1998) observed lipidic dendrimers in aqueous medium forming asymmetric fibre-like structures (Figure 1.9) and monolayer formation at the air/water interface of lipidic dendrimers.

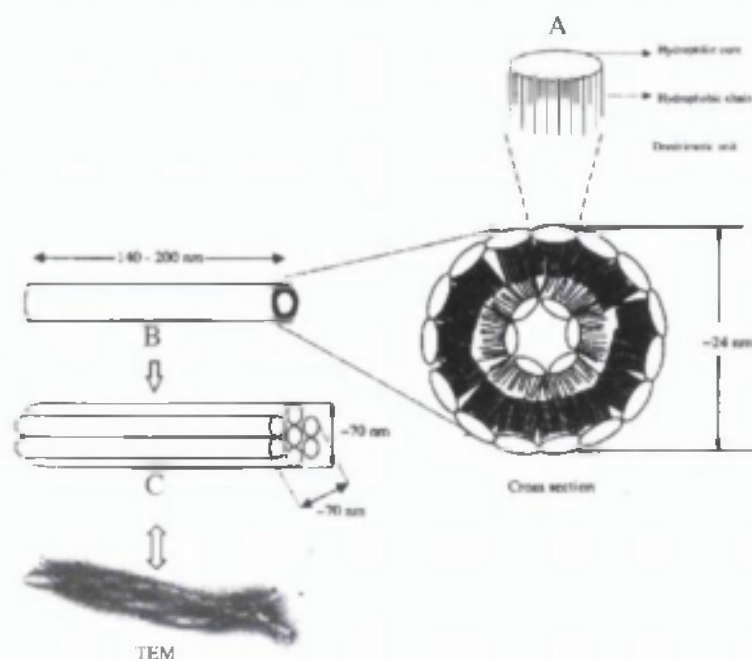


Figure 1.9. Possible models of self assembled lipophilic dendrimers (A) in aqueous dispersions. A typical tubule (B) with the dimensions of 140-200nm in length and 24 nm thickness with the possible association in filaments. (from Sakthivel *et al.* 1998)

More conventionally amphiphilic dendritic matter can assemble into micellar aggregates in aqueous solution (Gitsov and Fréchet 1993; Al-Jamal *et al.* 2005a). Dendron characteristics follow to a great extent conventional surfactant behaviour, where water soluble matter will form micelles, whereas poorly water soluble matter will form more stable vesicular structures (Al-Jamal *et al.* 2005).

1.4.1.3 Self-assembly of dendritic matter which can extended into macroscopic structures (3)

A schematic representation of possible self-assembly of dendritic matter which can be extended into macroscopic structures is shown in Figure 1.10.

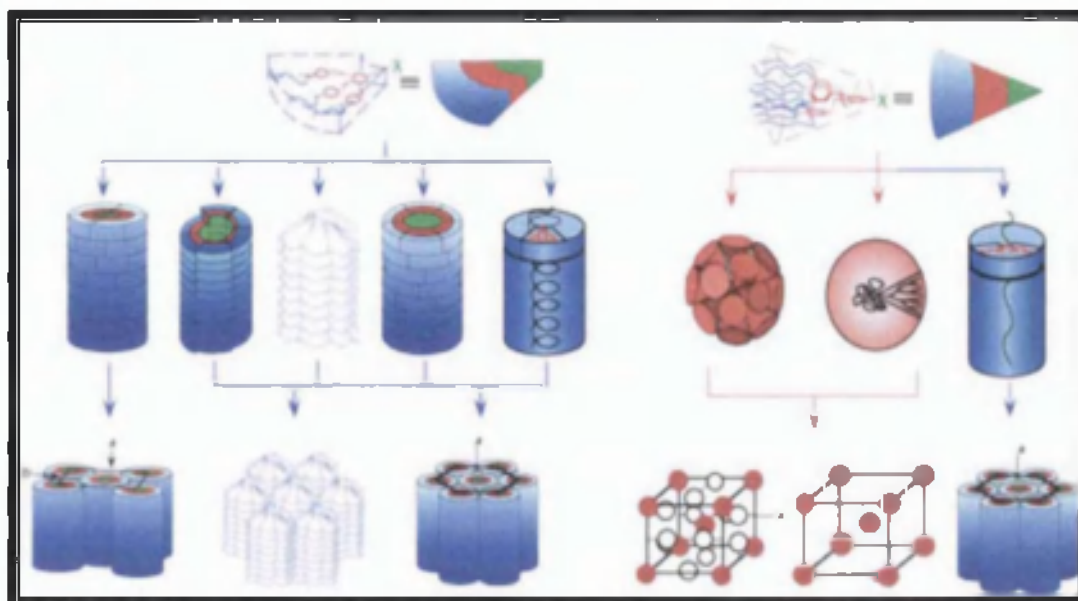


Figure 1.10. Schematic representation of the self-assembly of 12-ABG (3,4,5 tris(4 alkyl-1-oxy)benzyloxy benzoate) dendrons into supramolecular cylindrical shapes (left) and spherical (right) structures and subsequent formation of columnar hexagonal and cubic liquid crystalline assemblies, respectively (from Balagurusamy *et al.* 1997)

As shown, depending on the physical and chemical properties, the dendrons can arrange into an array of different structures from cylindric to spherical and then further arrangement of these into hexagonal, cubic structures or crystalline structures. Percec *et al.* (2000a,b) found that using Fréchet type dendrons with hydrophobically modified surface groups, the 1st and the 2nd generation dendrons assembled into cylindrical structures which then packed hexagonally, while the 3rd generation dendron assembled in a spherical structure and packed in a cubic manner.

As mentioned above self-assembly of a multitude of dendrons and dendrimers is extensively studied. However, the structures formed after interaction with drugs or DNA is also of interest as diameter, shape and structural arrangement most probably will affect flow properties and interaction with the targeted areas. One would expect to entrap drugs inside the voids the dendritic structures and therefore only to a lesser extent affect the assembly of these. However, one of the most extensively studied is dendritic molecule-DNA assembly, with elongated oval shaped formation

(dendriplexes) obtained from lysine based dendrimers and DNA (Ramaswamy 2004) (Figure 1.11).

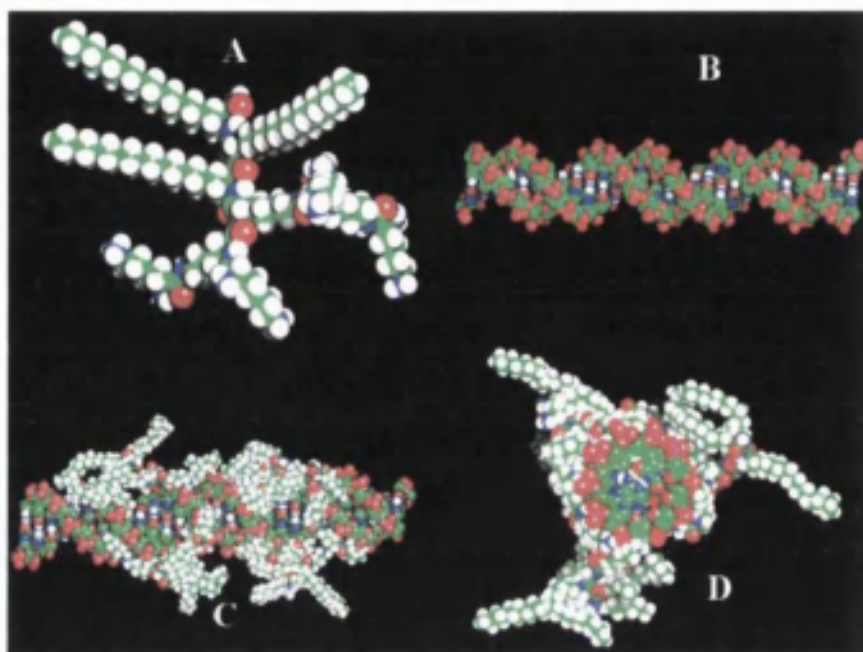


Figure 1.11. Molecular model of dendriplexes using a 32 base pair segment of DNA. (A) lysine based dendron $(C_{18})_3Lys_7(NH_2)_8$, (B) DNA. (C) and (D) two different orientations of the dendriplex (Ramaswamy 2004).

Self-assembly of dendritic matter is in itself an interesting concept, however without potential applications it is of no use. The potential of dendritic matter is discussed in the following section.

1.5 Dendrimer applications

The full potential of dendrimers, *per se*, is not to be underestimated. Recently association of dendritic matter was controlled to mimic protein structures. Percec *et al.* (2004) assembled amphiphilic dendritic dipeptides into helical pores which mimic the structure of natural pore forming proteins. Because a close link is found between protein function and protein structure assemblies of dendritic matter will allow a

design of a variety of biological inspired systems. The full potential of these systems is still to be studied.

Several approaches, such as site-specific delivery systems, chemical modification of peptides, bioadhesive systems, and concomitant administration of penetration enhancers or protease inhibitors have been investigated for oral delivery of peptides. The use of dendritic systems may also be a way to improve delivery via the oral route. Until now most studies have been carried out *in vitro* using intestinal model systems (Wiwattanapatapee *et al.* 2000). As mentioned the preferred administration of drugs is via the oral route. Entrapment of peptides and proteins in dendritic structures might help improve their stability in gastric and intestinal fluids as well as improve poor absorption in the gastrointestinal tract (GIT). In the case of therapeutic compounds, the molecules have to remain intact when they reach the systemic circulation and therefore stability is of importance. Biodistribution of dendrimers after oral administration by itself, as controlled aggregates and dendrimer-drug conjugates needs to be more thoroughly investigated if dendrimers are to be successful as carriers. The stability, drug interaction, interaction with lipidic membranes and flow properties also needs to be studied. However, Jevprasesphant *et al.* (2003) showed that surface engineering of PAMAM dendrimers reduced cytotoxicity and enhanced the rate of transport of dendrimers across epithelial cells.

At the moment the field is possibly still in its infancy and the current understanding of how dendrons and dendrimers interact, e.g. with bilayer membranes (Zhang *et al.* 2000), liposomes (Purohit *et al.* 2001), colloidal gold (Garcia *et al.* 1999, Singh *et al.* 2003), drugs such as piroxicam (Wiwattanapatapee *et al.* 1999), 5-fluorouracil (Khopade *et al.* 1999), ibuprofen (Milhem *et al.* 2000), indomethacin (Liu *et al.*

2000) and penicillin G (Al-jamal *et al.* 2003) are the first step in order to optimise delivery systems. Once a complete understanding of the mechanisms involved is reached the potential applications are multiple- vaccine delivery, gene delivery, drug delivery and generally in materials science. The nature of interaction is naturally dependent on the functional groups of the dendrimers and the size, shape, charge and hydrophobic/hydrophilic nature of the vehicle.

1.5.1 Stability of dendrimer complexes

Incorporation, encapsulating or surface adsorbing peptides in polymeric structures have all been shown to protect against degradation by the substances found in the gastrointestinal tract (GIT) (Allemann *et al.* 1998). Studies have shown that adsorption of plasmid DNA onto mineral surfaces (Romanowski *et al.* 1991) such as clay minerals (Demaneche *et al.* 2001) protects the DNA against degradation. Furthermore, studies have shown that adding dendrimer to DNA, protects the plasmid DNA against nuclease activity (Toth *et al.* 1999; Bielinska *et al.* 1997).

The area has still not been thoroughly studied and therefore it is still not known if all classes of dendrimers have a nuclease protection effect. Two hypotheses have so far been proposed to explain the protection mechanism. First, that certain particles such as dendrimer and clay could be considered as providing plasmid DNA physical protection from nuclease. The second and an alternative hypothesis, proposed by Demaneche *et al.* (2001) is that nuclease itself adsorbs onto the clay thus reducing its enzymatic activity. Adsorption would physically separate plasmid DNA and the nucleases on the clay surfaces. This however needs to be studied more thoroughly in order to fully exploit the possible use of dendrimers for oral gene delivery.

Only a few studies have been made on the physical properties of dendritic complexes in cell culture media. One such study by Pouton *et al.* (1998) showed that cationic poly (amino acid)/DNA complexes when diluted into opti-MEM media, were unstable to electrolyte challenge. The particle size increased and it was suggested that the particle growth might have represented aggregation due to changes in the electrical double layer by lowering the zeta potential, compressing the electrical diffuse layer, leading to reduced charge repulsion between the particles. Another paper by Singh *et al.* (2003) investigated the effect of physiological media on the stability of surface-adsorbed DNA-dendron-gold nanoparticles. Whilst gold-dendron-DNA nanoparticles maintained their original state in terms of size and surface potential, there was a rapid and drastic alteration in the size of the adducts when high concentrations of salt or cell culture media were used. This flocculation would not only decrease release of drug/DNA but would also affect particulate uptake of the carrier.

Not only electrolytes affect particles. Proteins such as serum albumin has been shown to bind to poly-L-lysine/DNA complexes forming a ternary poly-L-lysine/DNA/albumin complex (Dash *et al.* 1999). This indicates that cell culture media, which contains a wide range of salts, hormones, growth factors, vitamins and serum proteins is a complex media and there are therefore a multitude of factors that determine the physical properties of the complexes. Therefore, characterising drug delivery systems in the presence of relevant physiological fluids and media prior to *in vivo* studies is important, and may be one reason that *in vivo* and *in vitro* correlations in gene transfection studies are so limited.

1.5.2 Gene delivery

Although the formation of dendriplexes (complex formed between cationic dendrons and polyanions) has been characterised (Ramaswamy *et al.* 2003), transfection *in vitro* in a range of cell lines has only shown a limited degree of success (Toth *et al.* 1999, Shah *et al.* 2000, Bielinska *et al.* 2000). Therefore efforts have been made to optimise the transfection efficiency. Toth *et al.* (1999) used nuclear localization signal (NLS) sequences in pursuit of this, but did not find any enhanced transfection efficiency, possibly because of steric hindrance and aggregation of the complexes. More studies need to be conducted with NLS sequences to determine the effect of these. However, Qiagen has two transfecting agents on the market, namely SuperFect and PolyFect (Tang *et al.* 1996) and therefore the potential for utilising dendrimers as carriers for gene delivery definitely exist and is an area which is currently under investigation.

Although the mechanism for dendrimer-based transfection remain obscure, it is likely that a variety of parameters such as plasmid DNA size and structure (whether the DNA is circular, linear or supercoiled), the chain length and position of the lipid (for cationic lipidic dendrimers), the dendrimer/DNA ratio and the resulting size of the complex, the composition of the bulk medium, storage time and conditions, modes of administration and target cell types may determine the extent of transgene expression (Toth *et al.* 1999).

1.6 Aims and objectives for the thesis

This thesis describes the synthesis and characterisation of lipidic lysine based dendrimers and the study of controlled aggregation of these dendrimers into what we term dendrimer-derived nanoparticles. The cytotoxicity and haemolytic activity in selected cell lines *in vitro* was then studied and prior to oral uptake and translocation studies of these nanoparticles, the stability of these were assessed in relevant biological media.

The aims of the work described in this thesis were as follows:

Aim 1: To synthesise various lipidic dendrimers using Fmoc solid phase peptide synthesis.

Aim 2: To formulate and study dendrimer-derived nanoparticles for oral uptake studies.

Aim 3: To assess *in vitro* dendrimer-derived nanoparticle cytotoxicity using Caco-2 and haemolytic activity in red blood cells.

Aim 4: To investigate the stability of dendrimer-derived nanoparticles in purified and simulated stomach and gastric fluid.

Aim 5: To assess the biodistribution of dendrimer-derived nanoparticles after oral administration.

Chapter two

Dendrimers synthesis

2.1 Introduction

Many different particulates have been assessed as carriers for oral administration. In the search of an alternative carrier which would potentially target Peyer's patches, dendrimers are an interesting prospect because of their versatility. The dendrimer architecture provides the basis for a variety of different structural designs through the control of composition, functionality and geometry. In our laboratory we designed a series of lysine based dendrimers with lipidic functionalities. By varying the generation as well as the length of the surface functionalised lipidic chains, dendrimers with various hydrophobicity and size were synthesised. These dendrimers can theoretically act as a delivery system for a multiplicity of different drugs, hydrophilic or hydrophobic. However, drug carrying potential is outside the scope of this thesis and therefore the dendrimer carriers alone have been studied in this thesis.

Over the years since their discovery as a class many different dendrimers have been synthesised, using either liquid phase peptide synthesis (LPPS) or solid phase peptide synthesis (SPPS). The approach used in our laboratories is SPPS and therefore will be the technique described in more detail in this thesis. SPPS was first proposed by R.B. Merrifield in 1962 with a full paper published in 1963. Since, the technique was widely unchanged for many years. In 1985 D. A. Tomalia first reported dendrimers synthesised using SPPS. Tomalia used an approach later termed as the divergent method. The divergent method (Tomalia *et al.* 1985) builds the dendrimers from the core to the surface (Figure 2.1). Starting from the reactive core, a generation is grown, and then the new periphery of the molecules is activated for reaction with more monomers. The other main synthetic approach developed for the synthesis of dendrimers was the convergent method (Hawker and Fréchet 1990), building the dendrimers from the exterior (Figure 2.1) of the dendrimers, and works as shown diagrammatically in Figure 2.1 gradually linking surface units together with more monomers. When the growing wedges are large enough, several are attached to a suitable core to give a complete dendrimer.

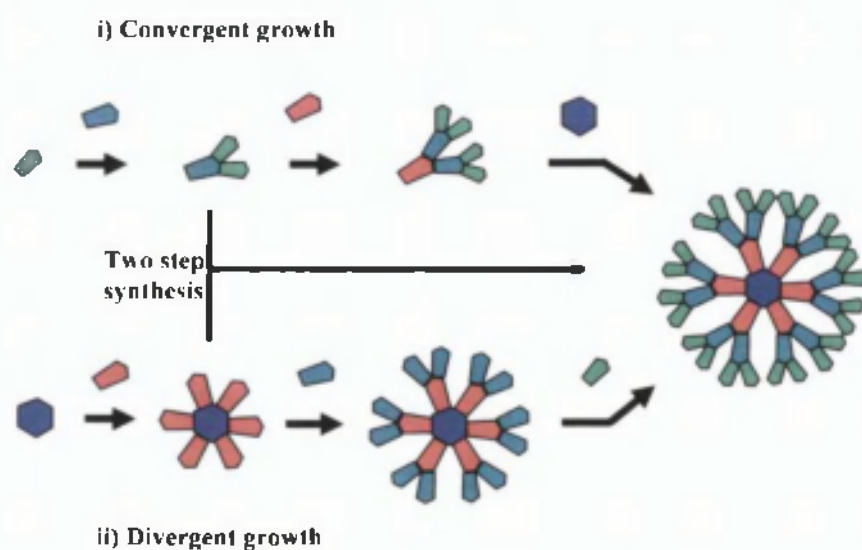


Figure 2.1. Shows the two basic approaches to dendrimer synthesis i) the 'convergent' growth (Hawker and Fréchet 1990) and ii) the 'divergent' growth (Tomalia *et al.* 1985).

Designing dendritic molecules through the selection of the cores, branching units, the linkages used to connect the branching units and also the synthetic method are of importance. Some of the components most commonly used for solid phase peptide synthesis and dendrimer synthesis are discussed here.

2.2 Solid Phase Peptide Synthesis

2.2.1 Principles of solid phase peptide synthesis

The principle of solid phase peptide synthesis is to expand a growing chain, peptide, oligonucleotide or other suitable oligomer, while it is attached to a stable solid particle. It remains attached to this particle throughout all the synthetic steps and is separated from soluble reagents and solvents by simple filtration and washing. Finally the product is detached from the solid support and purification and characterization is carried out.

The separation process is quick and simple and can be machine-aided. Compared to the corresponding operations in solution chemistry there is a significant time and labour advantage. Solid phase peptide synthesis, was a major breakthrough allowing for the chemical synthesis of peptides and small proteins. The general scheme of solid phase peptide synthesis is illustrated in (Figure 2.2).

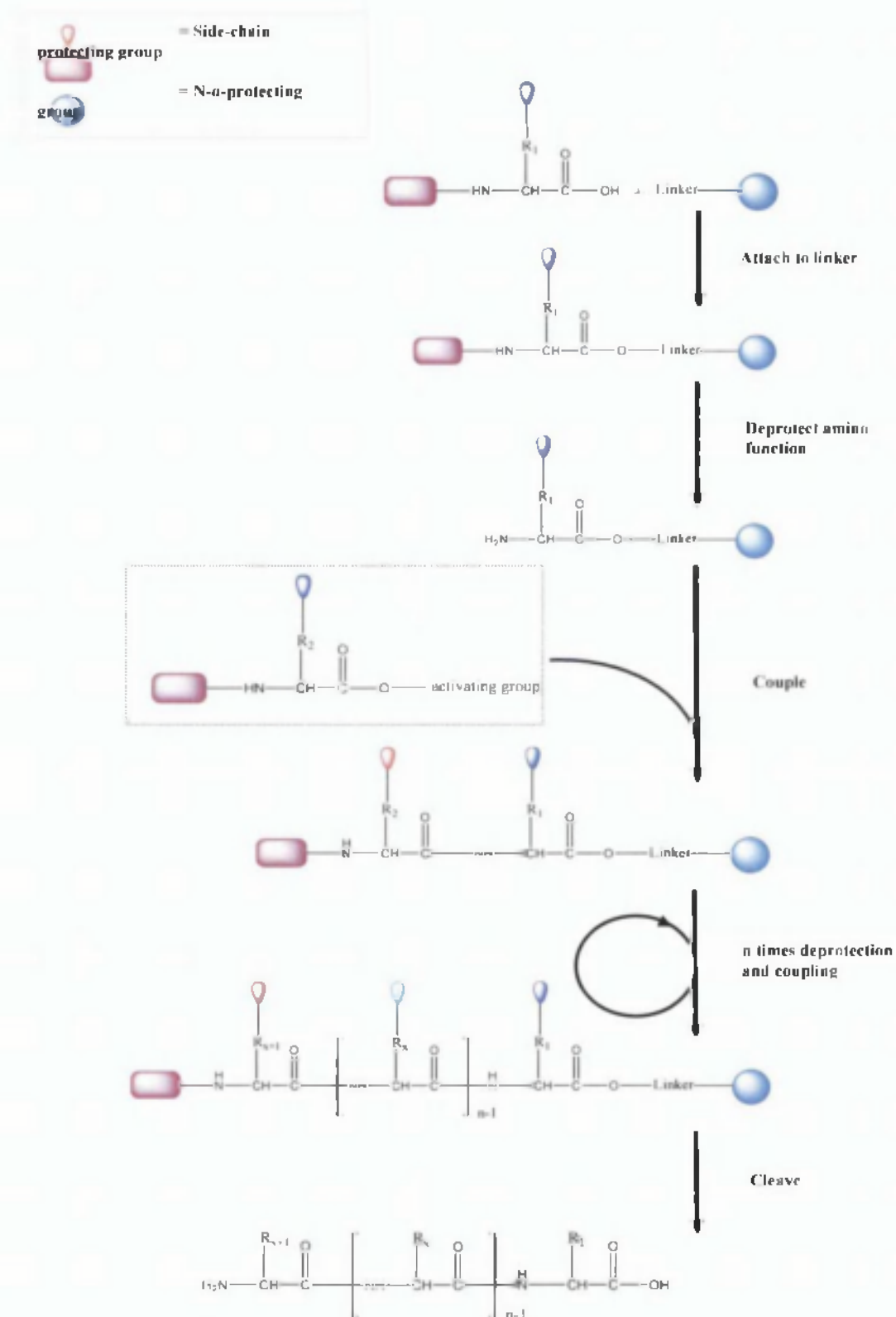


Figure 2.2. General scheme of Solid Phase Peptide Synthesis (SPPS) (Modified from Novabiochem 2004/5)

2.2.2 Solid supports

The solid support in solid phase peptide chemistry consists of a material that is insoluble, have good swelling properties in the main solvent used and chemically unreactive to all of the compounds utilized in the synthetic process. The most commonly used resins available are derivatives of divinylbenzene (1 % or 2 % w/w) cross-linked polystyrene. The most important part of the resin is the linker as this determines the functional group after cleaving the resin. Below some examples of linkers are listed according to their functional group after cleavage (Figure 2.3). The most widely used resin for Fmoc SPPS with a peptide amide linker is the *4-(2,4-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-methylbenzhydrylamine(Rink Amide MBHA resin)*. In this thesis *Rink Amide MBHA resin* was chosen for the Fmoc solid phase peptide synthesis, as it had excellent swelling properties and because one of the structural features of the dendrimers to be synthesised was an amide group in the core. The *Rink Amide MBHA resin* formed a amide after cleavage of the peptide from the resin.

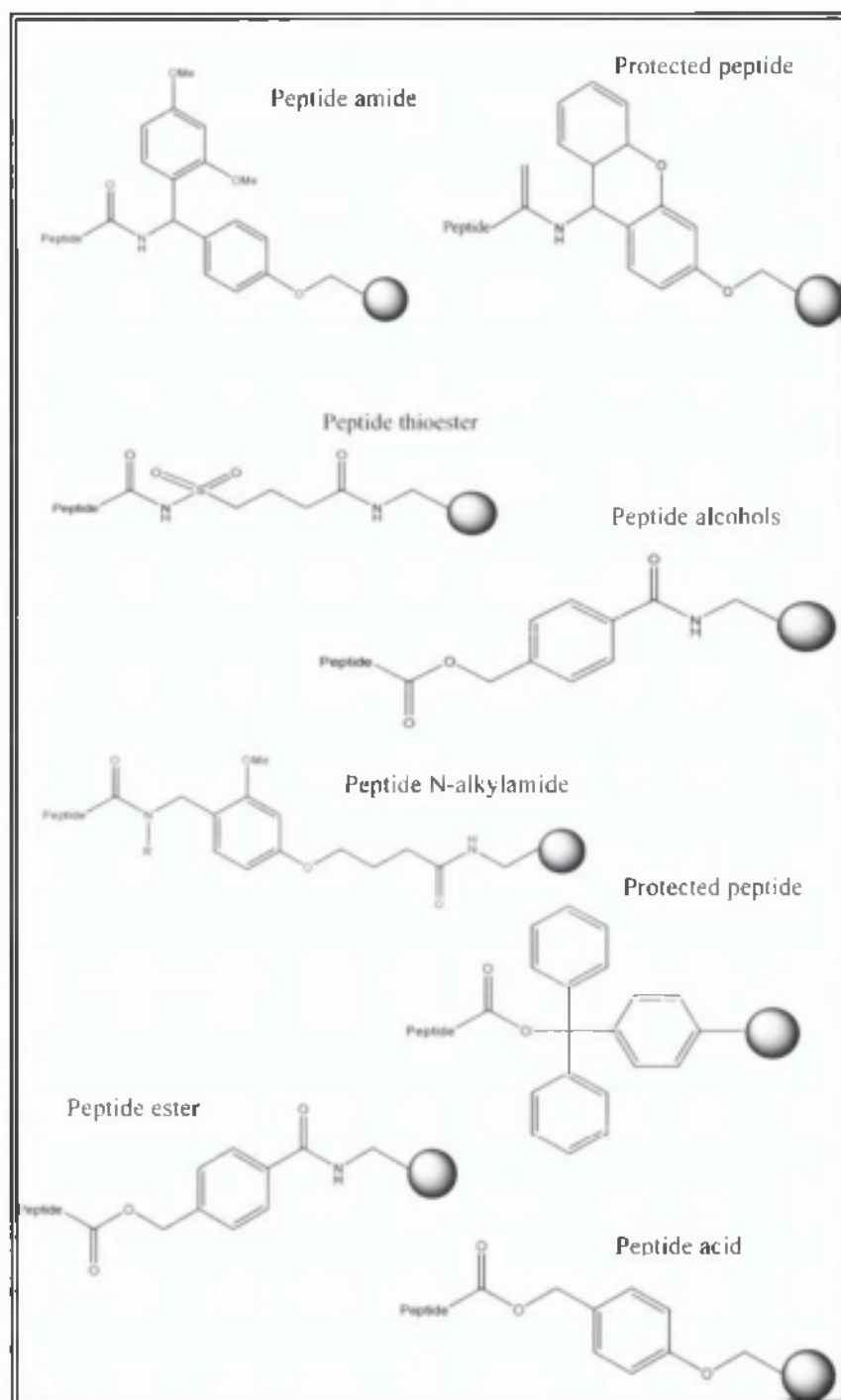


Figure 2.3. Examples of linkers listed according to their functional group after undergoing cleavage (Novabiochem 2003). Example of resins with a peptide amide linker, 4-(2, 4-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-methylbenzhydramine (Rink Amide MBHA) resin (used in this thesis), protected peptide amide linker, 9-Fmoc-amino-xanten-3-yloxy-chloromethylpolystyrene-divinylbenzene (Sieber Amide resin), peptide thioester linker, 4-Sulfamylbutyryl aminomethylated polystyrene (AM) resin, peptide alcohol linker, 4-hydroxymethylbenzoic acid (HMBA) 2-acrylamidoprop-1-yl-(2-aminoprop-1-yl) polyethylene glycol₈₀₀ and dimethylacrylamide cross-linked with his 2-acrylamidoprop-1-yl polyethylene glycol₈₀₀ (PEG₈₀₀), peptide N-alkylamide linker 4-Sulfamylbutyryl AM resin, protected peptide acid linker, 2-Chlorotrityl resin, peptide ester linkers, HMBA PEG₈₀₀ and peptide acid linker, *p*-benzyloxybenzylalcohol (Wang) resin.

2.2.3 Dendrimer cores

When designing dendrimers the core selected plays an important role as it may provide rigidity and conformational influence on the overall structure of the dendrimer (Sadler and Tam, 2002). Most cores employed for the synthesis of dendritic structures are simple small organic molecules with a molecular weight less than 1KDa. Most of these cores are commercially available which has enabled many research groups to study peptide dendrimers. A selection of core molecules are shown in Figure 2.4. The most common cores utilized for the synthesis of peptide dendrimers are simple amino compounds, such as amino acids and dipeptides. One of the most simple amino cores used for the synthesis of peptide dendrimers are of the ethylene diamine ammonia type. Heterocyclic compounds such as porphyrin and unusual amino acids have also been utilised (Sasaki and Kaiser, 1989). Some molecules do not have a distinct core as defined in the original concept of dendrimer synthesis by Tomalia *et al.* (1985). Molecules such as dendrons or 'partial dendrimers' have a starting point which may be termed the core of the dendritic structure (Ramaswamy *et al.* 2003). If these structures are then further branched they will result in a more spherical structure with the focal point being in the center of the molecule (Sakhtivel *et al.* 1998), giving a more obvious core to the dendritic structure. Several cores have been used which would assist folding into structures such as α -helices, β -sheets (Schneider and Kelly 1995, Jefferson *et al.* 1998). These cores are of interest in the design of artificial proteins.

In this thesis glycine was used at the focal point, termed the core, to which lysine was added as a branching unit to make up the dendritic structure.

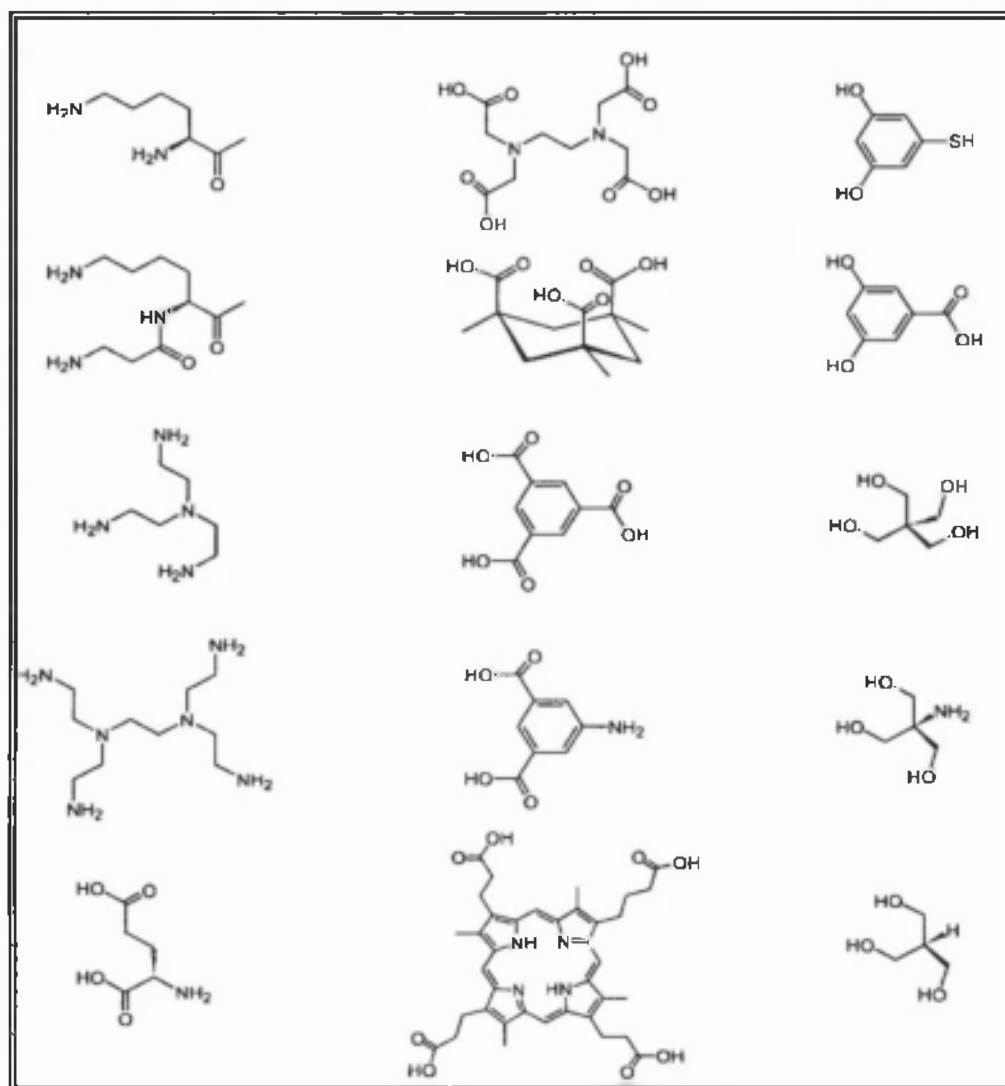


Figure 2.4. Structures of commonly used amino, carboxylic and hydroxylic core molecule utilized for the synthesis of dendritic structures (Sadler and Tam, 2002).

2.2.4 Side – chain – protecting groups

Solid phase peptide synthesis using the base labile α -amino protecting group, 9-fluorenylmethoxycarbonyl (Fmoc), was introduced in by Carpino and Han (1972). Generally in Fmoc SPPS the α -amino group is protected by Fmoc, while the side chain functionality is protected by the acid labile t-butyl type protecting groups. Fmoc-based SPPS provided an alternative to the t-Boc SPPS and has the advantage of using a milder acid for the cleavage process. α -Amino group deprotection of the Boc protecting group is achieved by trifluoroacetic acid (TFA) and the Fmoc

protecting group with the weak base piperidine. Final cleavage of the peptidyl resin and side chain deprotection requires strong acid, such as hydrogen fluoride (HF) or trifluoromethanesulfonic acid (TFMSA), in the case of Boc chemistry and TFA in Fmoc chemistry (Chan and White 2000). Dichloromethane (DCM) and N, N-dimethylformamide (DMF) are the main solvents used for resin deprotection, coupling and washing (Novabiochem 2004/5). The side chain protecting group defines the synthesis procedure used and therefore either Fmoc SPPS or Boc SPPS can be utilised. For the synthesis of lipidic dendrimers in this work, Fmoc SPPS was used.

2.2.5 Formation of the peptide bond, coupling

In recent years, activating reagents have become widely used because of their catalysing properties even between sterically hindered amino acids, and their general lack of side reactions. Most are based on phosphonium, aminium or diimide salts which in the presence of a tertiary base (N-ethyldiisopropylamine, DIEA) can convert protected amino acids to a variety of activated species. The most commonly used, (benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate (BOP), phosphonium hexafluorophosphate (PyBOP) (Martinez *et al.*, 1985) and N-[(1H-benzotriazol-1-yl)(dimethylaminomethylene)]-N-methylmethanaminium hexafluorophosphate N-oxide (HBTU), 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and 1-hydroxybenzotriazole (HOBt) (Knorr *et al.*, 1989) (classification and chemical name stated in Figure 2.5). Aminium-based activation reagents should not be used in excess relative to the carboxylic acid components as this can lead to capping of amino terminus through

guanidine formation (Gausepohl *et al.*, 1992). HBTU was chosen as the coupling reagent for the synthesis of all dendrimers in this work.

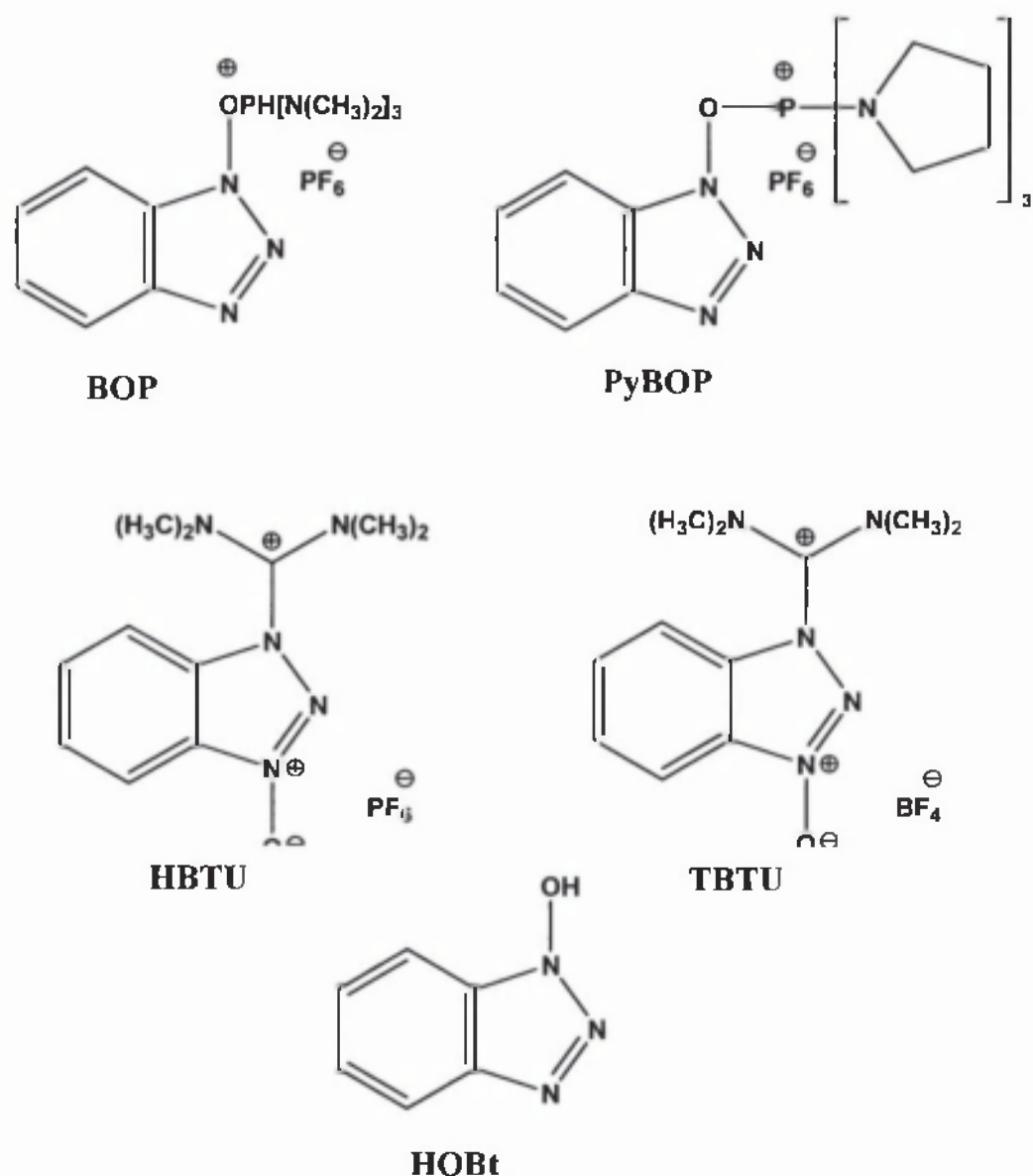


Figure 2.5. Different classes of some commonly used coupling reagents. 1-hydroxybenzotriazole (HOBT). Aminium type coupling reagents: N-[(1H-benzotriazol-1-yl)(dimethylaminomethylene)]-N-methylmethanaminium hexafluorophosphate N-oxide (HBTU) and 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU). Phosphonium-type coupling reagents: (benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate (BOP), phosphonium hexafluorophosphate (PyBOP).

The formation of an amide bond between two amino acids requires energy. Currently, activation of the carboxyl group remains the principle of all coupling methods in use. Efficient peptide-bond formation requires chemical activation of the carboxyl component of the N- α -protected amino acid. Conversion of the carboxylic acids to a powerful acylating agent is achieved by substitution of the hydroxyl group for an electron withdrawing substituent which polarises the carbonyl group and renders its carbon atom sufficiently electrophilic to facilitate the nucleophilic attack by the amino group. A tetrahedral intermediate is formed and is stabilised by the elimination of the electron withdrawing substituent.

2.2.6 Peptide chain assembly

Peptide chain assembly is possible because the peptide is covalently linked to a solid support. Once swollen in an appropriate solvent, the solid support provides an interpenetrating polymer network, within which the synthesis can take place. The first step in peptide chain assembly is removing the Fmoc protecting group from the α -amino group using 20 % (v/v) piperidine in DMF. In the case of incomplete Fmoc deprotection, which can occur for long peptides even in presence of high concentrations of piperidine (Fontenot et al., 1991), the time required for deprotection can be increased (from 20 min to an appropriate time) or a stronger base such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) with 2% piperidine can be used. The resulting trifluoroacetate salt of the peptide resin is then neutralised with the hindered tertiary amine diisopropylethylamine (DIEA). The use of highly purified tertiary amine helps minimise side reactions (Tam *et al.*, 1979). The activated amino acid is then added to form a peptide bond. In this thesis DIEA was used to neutralise

the trifluoroacetate salt and the polar solvent dimethylformamide (DMF) (Applied Biosystems, Cheshire, UK) was utilised as the swelling solvent as it maximises solvation and rapid coupling.

2.2.7 Monitoring reactions

During solid phase peptide synthesis, intermediates can be removed by repeated filtration and washing by organic solvents. The purity of the compound depends on the efficiency of the stepwise washing. Therefore each reaction involved in the peptide chain assembly should be monitored until the synthesis is completed. The information obtained at each step of the chain assembly is important so that the chemistry can be optimised. Monitoring the efficiency of the chain assembly is of importance to ensure the synthesis of a molecule of high purity and known structure.

The efficiency of coupling and deprotection steps of the chain assembly was evaluated utilising the resin test known as the 'Kaiser' or 'ninhydrin' test (Kaiser *et al* 1970). This test is a simple, quick, sensitive and accurate test (>99.9%) with good reproducibility. This is the most widely used qualitative test for the presence or absence of free amino groups (deprotection/coupling).

The Kaiser test (ninhydrin test) comprises of 3 different solutions:

- i) 80 g liquefied phenol (purity, 99.5%) dissolved, by heat, in 20ml absolute ethanol.
- ii) 2ml of a 0.001 M aqueous solution of potassium cyanide plus 98 ml pyridine (purity, 99%).
- iii) 5 g of ninhydrin (purity, 99%) in 100 ml ethanol (purity, 95%).

Following deprotection of the amines with 20% piperidine and washing with DMF, a minute amount of the peptide-resin sample are taken and washed several times (five or six) with DCM/MeOH (1:1 ratio) and transferred to a small glass tube. Two drops of each solution are added to the sample and the tube placed in an oil bath at 110°C for 5 min. 5ml 60% ethanol is then added to the tube and the colour observed. A dark blue coloured solution indicated the presence of free primary amino groups. The same method was utilised to monitor the coupling of Fmoc protected amino acids. A clear solution indicated that all free amino groups had undergone coupling. For the comparison a blank sample was prepared using the ninhydrin reagents and 60% ethanol. It, however, should be noted that some deprotected amino acids do not show the expected dark blue colour typical of free primary amino groups (Fontenot et al., 1991). Glycine and lysine which were used for the dendrimer synthesis in this thesis did show the expected dark blue colour and therefore the ninhydrin test was utilised to monitor the coupling and deprotection reactions during the dendrimer synthesis.

2.2.8 Cleavage from the resin support

Prior to the cleavage of the resin, any N-terminal Fmoc groups must be removed using piperidine. The peptide should then be thoroughly washed, particularly when DMF is used during synthesis because it is non-volatile and because residual basic DMF can have an inhibitory effect on TFA-acidolysis (Novabiochem 2004/5). After washing with DMF several times, the peptide resin should be washed with DCM several times to get rid of residue DMF and to shrink the resin. The peptide resin was then dried under high vacuum for 4h or overnight over potassium hydroxide (KOH).

The most favourable cleavage conditions depend on the individual amino acid residues present, their number and sequence, the side-chain protecting groups, and the type of linker attached to the resin. Approximately 10-25 ml TFA/g resin (95% TFA, and 5% deionised water or alternatively 95% TFA, 2.5 % water and 2.5% scavengers (triisopropylsilane, TIS) should be used to cleave the peptide from the resin. Scavengers are useful as they can react with the linker of the resin to prevent side reactions, especially important when the product contains nucleophilic groups susceptible to alkylation by the linker (Fields *et al.* 1990). The cleavage time and hence the reaction time varies depending upon the choice of amino acid building block and scavengers used. Therefore optimisation of the cleavage conditions before use is essential. The cleavage reaction should be left at room temperature with occasional stirring, the reaction time is dependent on the peptide sequence. The resin is then filtered under pressure and washed again with TFA. The filtrates are combined and 8 to 10 fold volume of cold ether was added drop-wise. Sometimes it is necessary to evaporate most of the TFA to achieve a good precipitation of the crude peptide.

The dendrimers utilised in this thesis were synthesised using Fmoc solid phase peptide synthesis. Small changes were made in the synthetic procedure and will be discussed where appropriate. Using the divergent approach the dendritic structures aimed to be synthesised are shown in Figure 2.6.

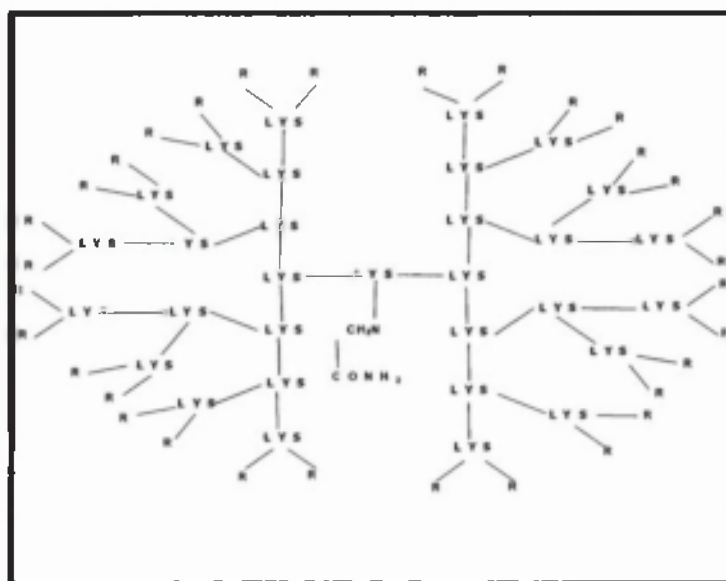


Figure 2.6. The general formula of lipidic polylysine dendrimer, R indicating either butyric acid (I), decanoic acid (II), dodecanoic acid (III), lys-(butyric acid)₂ (IV), lys-(decanoic acid)₂ (V) or lys-(dodecanoic acid)₂ (VI).

Glycine was used as the focal point and lysine coupled to make up a branched molecule. At the 5th and 6th lysine generation, lipidic chains (C₄, C₁₀ or C₁₂) were coupled to the external amino groups which result in dendrimers with surface hydrophobicity. These highly hydrophobic materials were constructed because hydrophobic particles are taken up more readily by Peyer's patches of the GALT (chapter 1, section 1.3.7). The dendrimers have been synthesised towards the use in the field of oral delivery.

2.3 Materials and Methods

2.3.1 Materials

Rink Amide MBHA resin (substitution 0.78mmol/g) and Fmoc-Gly-OH was purchased from Calbiochem-NovaBiochem UK Ltd, Nottingham, UK. Fmoc-lys(Fmoc)-OH was purchased from Advanced ChemTech Europe Ltd, Cambridgeshire, UK and HBTU (O-Benzotrizol-N, N, N¹, N¹- tetra-methyl-uronium-hexafluoro-phosphate from Severn Biotech Ltd, Worcestershire, UK. DCM (dichloromethane), ethanol, methanol and ether purchased from BDH, Leicestershire, UK and DMF (N,N dimethylformamide) obtained from Rathburn Chemicals Ltd, Walkerburn, UK. TFA (trifluoroacetic acid) was obtained from KMZ Chemicals Ltd, Surry, UK and glacial acetic acid, phenol, potassium cyanide, pyridine were all purchased from Aldrich, Dorset, UK and ninhydrine 99% from Avocado Research Chemicals Ltd, Lancashire, UK. DIEA (N-Ethyldiisopropylamine), piperidine, butyric acid, decanoic acid, dodecanoic acid and Fluorenylmethylsuccinimidyl (Fmoc) were obtained from Lancaster Synthesis Ltd, Lancashire, UK. Dodecanoic acid (Carboxy-¹⁴C) 0.8 mg was purchased from Sigma-Aldrich, Dorset, UK and tritium labelled lysine ([4,5 ³H] L-lysine 1 mCi in 1,0 ml sterile H₂O) purchased from Moravsek Biochemicals, CA, USA. All materials were used as purchased without further purification.

2.3.2 Methods

2.3.2.1 Dendrimer synthesis

Dendrimers with lipidic surfaces were synthesised using a solid phase procedure on a Rink Amide MBHA resin (loading: 0.78mmol/g). Each generation of the dendrimers

was synthesized using a three fold excess of HBTU and DIEA activated Fmoc-aminoacids in DMF (glycine as “core” and lysine as branching unit). 20% piperidine in DMF were utilized to achieve Fmoc deprotection.

The coupling and deprotection of amino acids was monitored using the ninhydrine test. A few resin beads were washed 4 to 5 times with 1:1 DCM:methanol. 1 drop of a 76% w/w phenol in ethanol, 2 drops of a 0.2mM potassium cyanide in pyridine and 1 drop from a 0.28M ninhydrine in ethanol were then added to the resin beads. The sample was heated on an oil bath at 110°C for 5 min after which 4ml of 60% aqueous ethanol was added. Free amino groups (deprotected) were indicated by the blue coloured solution and no free amino groups were indicated by a clear solution.

The 5th and 6th generation the terminal amino groups of the lysine were coupled to butyric acid, decanoic acid and dodecanoic acid. The carboxylic acids of the lipidic chains were activated using HBTU as a coupling reagent and DIEA as activating reagent. Again the reaction was monitored using the ninhydrine test

When the dendrimer synthesis was completed, the resin bound dendrimer was washed with DMF and DCM several times (5-6) and dried over vacuum for 4h. The resin was placed in a round bottom flask and 25ml TFA (95% v/v TFA, 5% v/v water) per gram resin was added. The solution was left for 1.5h with occasional stirring. The resin was removed through filtration and washed again with TFA. The filtrates were collected and the TFA was evaporated using rotary evaporation. Cold ether was added drop-wise and left overnight in the freezer. The ether was then evaporated and acetic acid (100 %) added. Double distilled deionised water was then added to the solution until precipitation was evident and the sample left overnight at -

4°C. The sample was then frozen down using liquid nitrogen and freeze dried. The compound was then weighed and collected.

2.3.2.2 Synthesis of radiolabelled dendrimers

2.3.2.2.1 Fmoc protection of radiolabelled lysine

1 g of tritium labelled lysine was dissolved together with sodium carbonate (1.46g) in water (17.8ml). Fluorenylmethylsuccinimidyl carbonate (4.4g, 6.70mmol) dissolved in acetone (17.8ml) was added in portions over a period of 60 min. The briskly stirred solution was kept at pH 9-10 by addition of 1 M sodium carbonate. After stirring overnight the acetone was evaporated. Ethyl acetate (2 x 20ml) was added and the mixture acidified with 2M hydrochloric Acid. The ethyl acetate layer was separated, washed with water (4 x 20ml), dried over anhydrous magnesium sulphate and evaporated to approximately one third volume. The product crystallized on addition of hexane.

2.3.2.2.2 Synthesis of radiolabelled lipidic dendrimer, GlyLys₃₁(C₁₂)₃₂,

Lipidic dendrimers (GlyLys₃₁(C₁₂)₃₂) were synthesised using Fmoc solid phase peptide synthesis as described in section 2.3.2.1. The only alteration in the synthesis procedure was at steps where Fmoc-amino acid lysine (branching unit) and the lipidic chains (dodecanoic acid) were added. The lysine were combined with tritium labelled Fmoc-[4,5 ³H] L-lysine(Fmoc)-OH before addition and the dodecanoic acid combined with carboxy-¹⁴C labelled dodecanoic acid. The mixtures were then coupled as described in section 2.3.2.1. The radiolabelled lysine solution (1 ml) was divided into four solutions (0.25 ml), and added with the 1st, 2nd, 3rd and 4th lysine generation. Initially several attempts were made to synthesise a dual labelled dendrimer, however, when the radioactivity of the synthesised dendrimer was

measured, the tritium was not detectable. The tritium label did not seem to be stable during the synthesis procedure and therefore the synthesised dendrimer (GlyLys₃₁(C₁₂)₃₂) was only carbon labelled.

2.3.2.3 Mass spectrometry

Structural control of dendrimer synthesis was predominantly done through mass spectroscopy. These methods only give structural conformation of the synthesised product, and cannot be used to assess the purity of compounds. Both electrospray mass spectrometry (ES-MS) and Matrix Assisted Laser Desorption Time of Flight (MALDI-TOF) Mass Spectrometer were used to obtain the mass spectra of compounds synthesised.

2.3.2.4 Electrospray ionisation mass spectrometry (ES-MS)

Mass spectra of Fmoc-[4,5 ³H] L-lysine(Fmoc)-OH was obtained using electrospray. ES-MS was obtained using a Finnigan Masslab Navigator quadrupole mass spectrometer under N₂ flow, 400 L/h; temp, 150°C; cone voltage 25V; capillary voltage 3V.

2.3.2.5 MALDI-TOF mass spectrometry

Mass spectra were run on a Applied Biosystems Voyager-DE-Pro Matrix Assisted Laser Desorption Time of Flight Mass Spectrometer (MALDI-TOF) operating in linear mode using a 337nm laser. 2-(4-hydroxy-phenylazo)-benzoic acid (HABA) was used as a matrix (10 mg/ml in methanol). HABA is especially suitable for non polar synthetic polymers (Voyager spectrometry workstation user guide). 4 µl of a dendrimer (I to VI) sample (dissolved in 20:80 acetic acid and DCM) was then mixed with 4 µl of matrix (HABA) and 1 µl of the mixture was loaded onto the

sample plate. The sample was allowed to dry until all solvents was evaporated and their spectrum determined.

2.3.2.6 NMR

The ^1H -NMR spectra of the lipidic lysine based dendrimers were acquired at a concentration of 1 mg/ml in 100 % d_6 -DMSO. The data points were obtained over a period of 3 h in the t_2 domain using a Bruker AVANCE 400 MHz spectrometer at 298 K and analysed by computer using Mestrec v459 software. Chemical shifts were referenced to DMSO.

2.3.2.7 High Performance Liquid Chromatography (HPLC)

The HPLC equipment utilised was an Applied Biosystems 400 solvent delivery system and Applied Biosystems 1480 injector Mixer (Applied Biosystems, Cheshire, UK). Solvent gradients were controlled by two microprocessor Gilson 302 single piston pumps. Compound absorbance was detected with an Applied Biosystems Programmable Absorbance detector and chromatographs were recorded on an LKB 2210 single channel chart recorder (Pharmacia Biotech Ltd, Herts, UK). All solvents were HPLC grade and filtered under vacuum through a 25 μm filter before running the solvents through the column. HPLC separation was carried out using a C_{18} column (3.5 μm , 2.1 mm x 50mm) obtained from Waters, Ireland. The solvent system used was 40: 40: 20, methanol, DCM and acetonitrile respectively. The flow rate was 0.6 ml/min and the retention time measured to 5.3 min.

2.3.2.8 Molecular modelling

Computer generated molecular models were constructed using the program RasMol, version 2.7.2.1. The models were optimised in vacuum for 3h using the optimisation program of the software. The molecular models were made with the assistance from Dr. S Haider from the department of Cancer research, London School of Pharmacy.

2.4 Results

As described the lipidic dendrimers were synthesised using Fmoc-solid phase peptide synthesis. Our group has up until this point, used Boc-solid phase peptide synthesis, however because of the dangerous toxicity of anhydrous HF, which has been used to cleave the peptide from the resin in Boc-chemistry, the alternative Fmoc-chemistry was used. Fmoc-chemistry does not require as harsh solvents as the alternative and therefore is preferable. The synthesised dendrimers will be described in the following section, along with examples of NMR, mass spectrometry and HPLC and a discussion of the obtained results.

2.4.1 Description of lipidic dendrimers

Lipophilic dendrimers were synthesised with glycine as the dendrimer core and lysine used as the branching unit. When the 5th and 6th generation of lysine were reached, lipidic chains (dodecanoic acid (C₁₂), decanoic acid (C₁₀) and butyric acid (C₄)) were coupled to the free lysine head groups, respectively 32 and 64 free terminal amino groups. The theoretical molecular weight of the dendrimers are

shown in Table 2.1 and a schematic representation of the dendrimers synthesised using Fmoc solid phase peptide synthesis shown below (Figure 2.7).

Table 2.1. Theoretically calculated molecular weights of the synthesised dendrimers

Number	Molecular formula	Molecular Weight (Daltons)
I	GlyLys ₃₁ (C ₄) ₃₂	6290.4
II	GlyLys ₃₁ (C ₁₀) ₃₂	8975.6
III	GlyLys ₃₁ (C ₁₂) ₃₂	9881.2
IV	GlyLys ₆₃ (C ₄) ₆₄	12590.8
V	GlyLys ₆₃ (C ₁₀) ₆₄	18019.1
VI	GlyLys ₆₃ (C ₁₂) ₆₄	19814.5

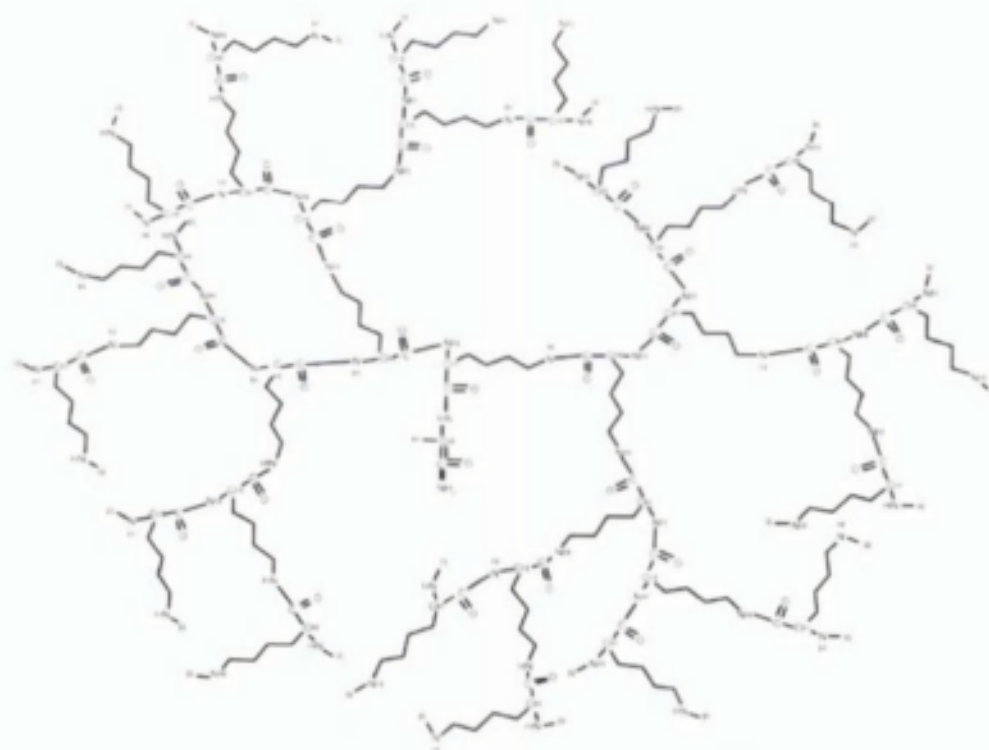


Figure 2.7. The formula of lipidic polylysine dendrimers synthesised. R indicating either lys-(butyric acid)₂ (I), lys-(decanoic acid)₂ (II) or lys-(dodecanoic acid)₂ (III) lys-lys₂-(butyric acid)₄ (IV), lys-lys₂-(decanoic acid)₄ (V) or lys-lys₂-(dodecanoic acid)₄ (VI) (Basic dendrimer structure taken from Al-Jamal *et al.* 2005).

Regarding the size and shape of lipidic dendrimers, experimental evidence such as molecular modelling suggests (the diameter was estimated from molecular modelling by measuring the distance from one side to another using a straight line), that the dendrimers are somewhat flat, floppy and oval shaped in the lower generations with a tendency to become more spherical at higher generations. The theoretical diameters of dendrimers I to VI are shown in Table 2.2.

Table 2.2. The theoretical diameter of dendrimers I to VI from molecular modeling.

Dendrimer	Horizontal, a, diameter, nm	Horizontal, b, diameter, nm
I	2.5	4.7
II	3.8	6.0
III	4.2	6.6
VI	3.7	5.5
V	5.0	6.7
IV	5.6	7.2

Because of the ellipsoidal shape of the dendrimers two diameters are stated. (a) states the short horizontal diameter and (b) the longer horizontal diameter. Using three dimensional molecular modelling, the diameters were estimated, by measuring the distance from one side to another using a straight line.

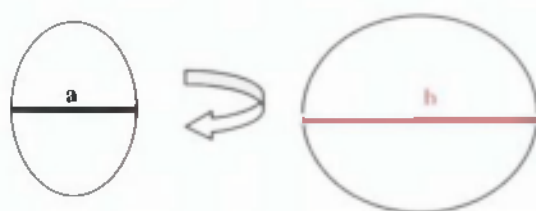


Figure 2.8 shows computer based models of the dendrimers IV, V and VI. The dendrimers have a very dense structure with either 31 or 63 lysine molecules forming the basic branch. With the addition of 32 or 64 lipidic chains respectively, the density of the structure is further pronounced. Even though the molecular models shows an overall very dense dendrimer structure, voids or cavities in the central part of the

dendrimer still exist. PANAM dendrimers (G7) for example, had cavities ranging from 0.5 to 1.5nm (Petkov *et al.* 2005). These cavities provide spaces for drug entrapment and an alternative to conjugation and adsorption of drug to the dendrimer. The size of the dendrimers are in the nanoscopic range, with diameters ranging from ~ 5nm to ~ 7nm. The diameter of the dendrimers increases by about 0.6 to 1.2nm per generation (Table 2.2).

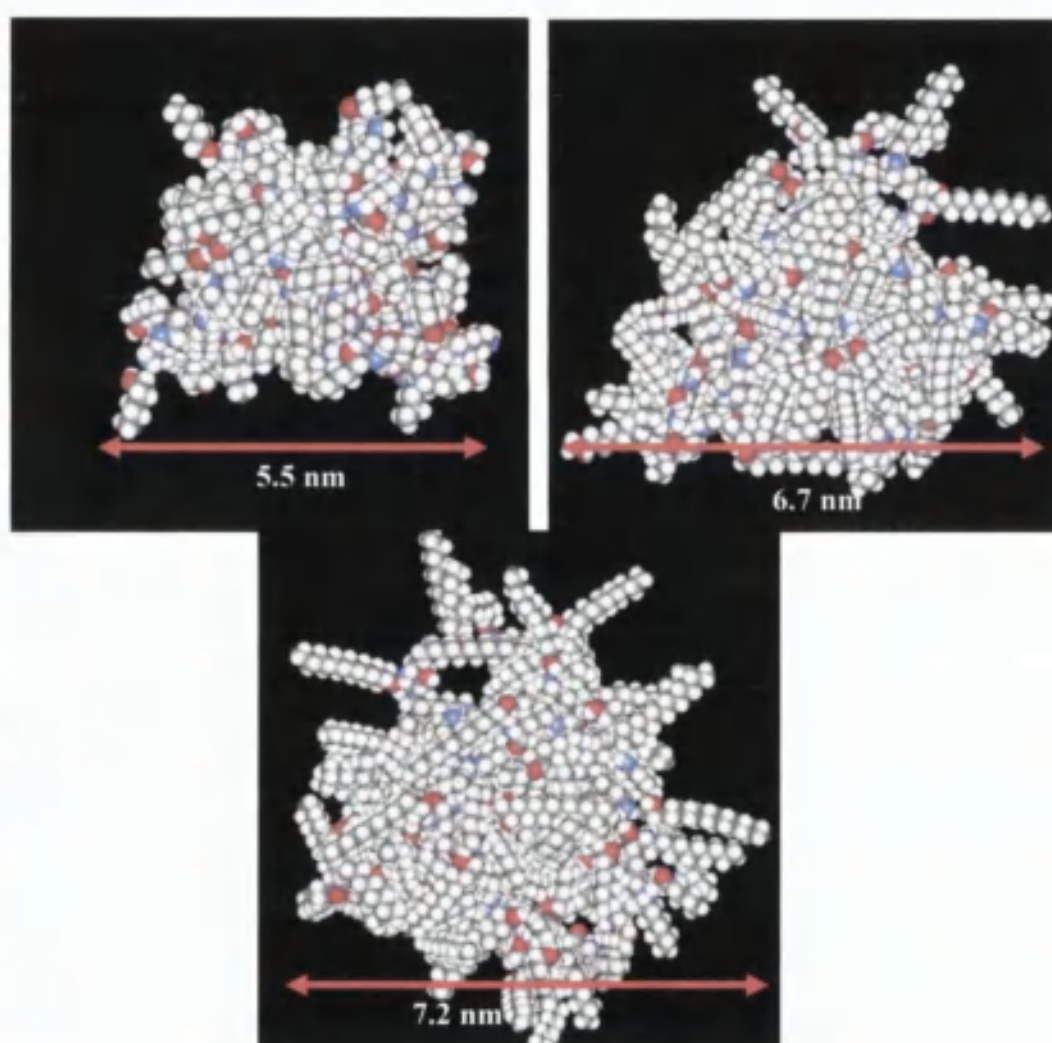


Figure 2.8. 3D images of lipidic dendrimers. In clockwise order, GlyLys₆₃(C₄)₆₄, GlyLys₆₃(C₁₀)₆₄ and GlyLys₆₃(C₁₂)₆₄.

2.4.2 Radiolabelled dendrimers

For the oral delivery studies (see chapter 5) because of the extent of work necessary one dendrimer was chosen (III, GlyLys₃₁(C₁₂)₃₂, *MW* 9865.2). Initially attempts were made to synthesise a dendrimer which was radioactively labelled in 2 separate positions. This approach was possible using tritium (³H) as one label and carbon (¹⁴C) as the second. A single label cannot determine absolutely, if the dendrimer, or any agent, is taken up intact or broken down and their fragments taken up. Dual labelling of the dendrimer would provide information regarding the integrity of the dendrimer after oral administration.



The blue * indicates tritium label and red * indicates carbon label.

Figure 2.9. The proposed tagging of the dendrimer.

Several attempts were made in order to achieve a dual labeled dendrimer. Figure 2.9, however non proved successful. The tritium label did not seem to be stable in the harsh solvent environment and could not be attached. Therefore, only a carbon labeled dendrimer (III, GlyLys₃₁(C₁₂)₃₂, *MW* 9865.2), indicated by the (*) in figure 2.9, was synthesized. The stability of the carbon label on the dendrimers was therefore determined in simulated gastric and intestinal fluid and will be discussed more fully in chapter 4. The ¹⁴C label dendrimer proved to be stable in simulated intestinal and gastric fluid. The label did not detach itself and the dendrimer broken down at to slow a rate to affect the absorption of the dendrimer in the Peyer's patches

of the GALT. Even though the dendrimer was not dual labelled, the stability of the label and the dendrimer ensured, that most of the dendrimers would be taken up as a complete particles.

2.4.3 Characterisation of lipophilic dendrimers

The dendrimers were characterized by, MS and HPLC while NMR was utilized to confirm the structural conformation of the dendrimer. From the NMR, a theoretical molecular weight could be calculated, which was confirmed by mass spectrometry. Mass spectrometry only confirms the molecular weight of dendrimers. The intensity of the peaks does not reflect the purity or the amount of dendrimer in the samples, only its presence. The problems faced using mass spectrometry on these compounds was the suppression of the signal by the lipidic chains of the dendrimers. Therefore the height of the signals should not be compared with the other signals. The other signals in the sample reflects impurities, which might be easily ionised, and therefore shows up as a comparatively more intensified peak. HPLC was then performed to assess the purity of the dendrimers. In the following section, the NMR and mass spectrometry findings are discussed. Using example, the actual molecular weight of the dendrimers will be determined, and are listed in Appendix 1.

2.4.4 NMR spectroscopy and mass spectrometry

For the assignments of NMR spectra the lysines could all be categorised as interior residues as they are branched together between glycine and lipidic chains. The interior lysine residues (1st, 2nd, 3rd, 4th, 5th generation lysine (I, II and III) and 6th generation lysine for dendrimers IV, V and VI) are characterised by two types of

amide bonds (H_{Nu} and H_{Ne}). Both these amides are involved in forming a peptide bond. The most external amide bonds of the dendrimer are coupled to lipidic chains, butyric acid, decanoic acid and dodecanoic acid. Any exterior lysine residues not forming amide bonds, incomplete reactions, will possess no N_N protons, but instead have NH_2 groups bound to either C_α or C_ϵ . The structure of the lysine with $H_{C\alpha-\epsilon}$ groups is shown in Figure 2.10.

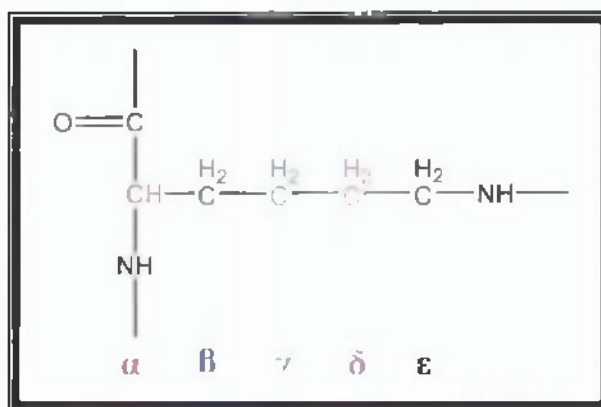


Figure 2.10. The structure of lysine with each proton sequence assigned its own colour and alphabetical number according to the position of the carbon.

The lipidic chains that occupy the exterior of the dendrimer are characterized by 3 different residues $-CO-CH_2-$, $-(CH_2)_x-$ and a $-CH_3$ group which assign for 3 specific proton peaks (Figure 2.12). An analysis and discussion of the NMR spectra of dendrimer II will be shown as an example. The results for the other dendrimers is listed with their assigned proton peaks in Appendix 1. Two different regions of the 1D proton NMR spectrum of dendrimer II in 100% d_6 -DMSO are shown in Figure 2.11 and 2.12.

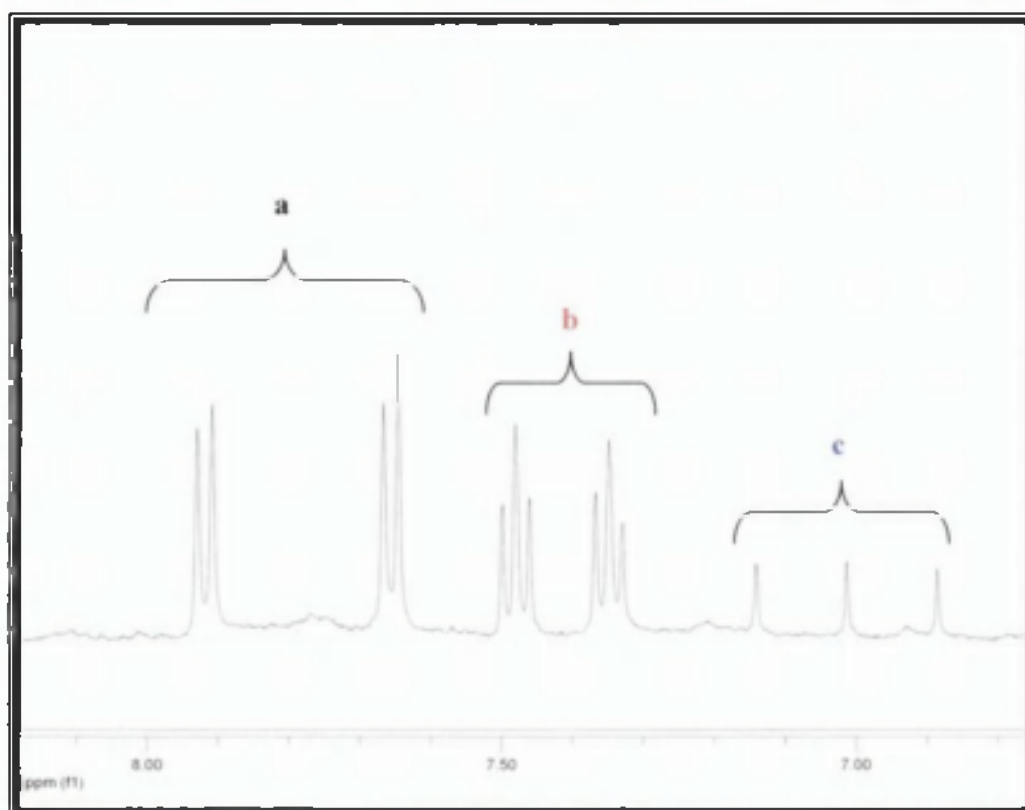


Figure 2.11. ^1H NMR spectrum of the H_N region of dendrimer II in 100 % d_6 -DMSO at room temperature. Two doublets which assign for $\text{H}_{\text{N}\alpha}$ protons, **a**, two triplets which assign for $\text{H}_{\text{N}\epsilon}$ protons, **b**, and three singlets, **c**, which assign for protons of glycine H_N .

As mentioned, the interior lysine moieties are characterised by two types of amide bonds $\text{H}_{\text{N}\alpha}$ and $\text{H}_{\text{N}\epsilon}$. As apparent in Figure 2.10, $\text{H}_{\text{N}\alpha}$ is next to a CH group and $\text{H}_{\text{N}\epsilon}$ next to a CH_2 group. All except the most exterior amide bonds, which are bound to decanoic acid, exist in a similar environment. Therefore two doublets ($\text{H}_{\text{N}\alpha}$, doublet, **a**) and two triplets ($\text{H}_{\text{N}\epsilon}$, triplet, **b**) are apparent in the amide region of the spectrum (Figure 2.11), which assigns for the 64 H_N protons, for details see appendix 1 under dendrimer II. Three singlets in the upfield region of the spectrum (**c**), are due to the amino groups attached to the glycine 'core' and assigns for 3 protons. However, if any amino groups exist, as a consequence of incomplete reactions, they will also appear in this region.

The lipidic chain of the dendrimer was clearly defined by 3 peaks in the spectra , Figure 2.12, a downfield peak of $-\text{CO}-\text{CH}_2-$ (d) followed by a peak for $-(\text{CH}_2)_7-$ (e) and then $-\text{CH}_3$ (f). Theoretically the $(\text{CH}_3)_{32}$ groups account for 96 protons, $((\text{CH}_2)_7)_{32}$ groups for 448 protons and the $(-\text{CO}-\text{CH}_2)_{32}$ groups for 64 protons.

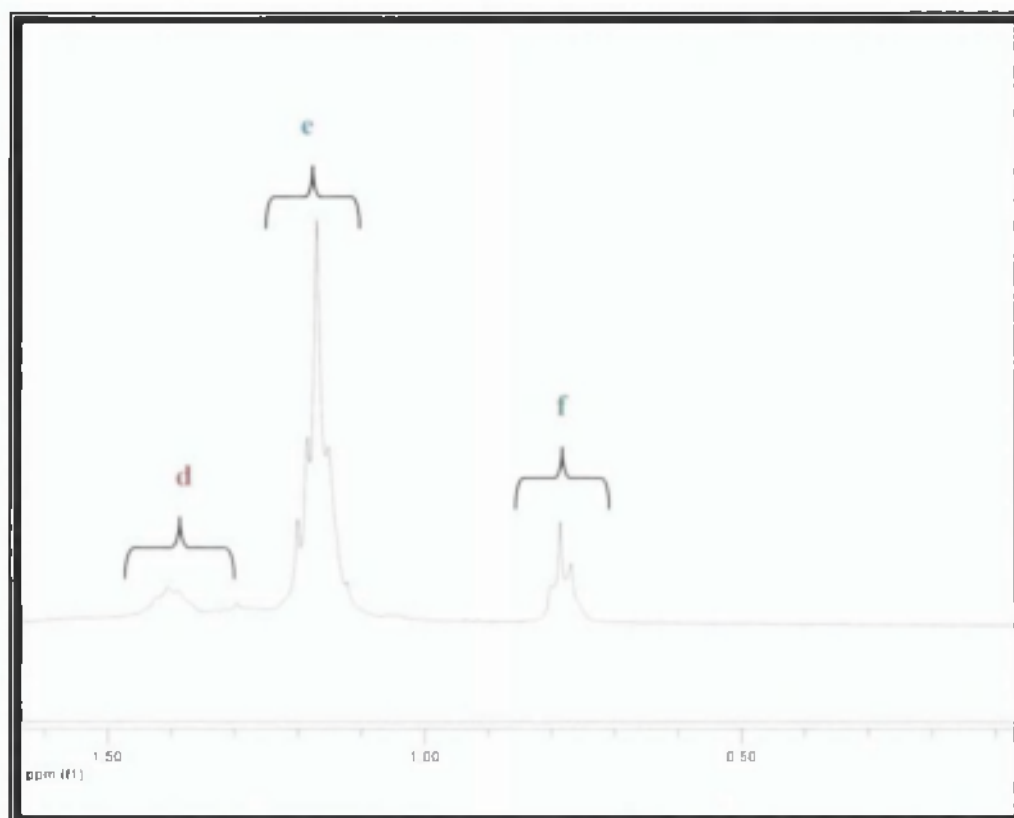


Figure 2.12. ^1H NMR spectrum of the H regions of decanoic acid of dendrimer II in 100 % d_6 -DMSO at room temperature.

The (H_C) protons of the lysine was scattered around 3 areas of the spectrum, from 1.7 ppm to 2.3 ppm, 2.8 ppm to 3.2 ppm and 4.0 ppm to 4.2 ppm. The assignment of protons however, was not clearly defined. Using the information from the H_N spectrum and the proton spectrum from the lipidic chain (decanoic acid), the structure of the dendrimer could be determined as follows:

The intensity of each peak in a spectrum accounts for a certain amount of protons, all depending on the structure of the molecule (Fessenden and Fessenden 1982). A perfect dendrimer (II) structure would have 1 glycine, 31 lysine and 32 C₁₀ groups, which in the ¹H NMR spectrum accounts for 5, 671 and 608 protons, respectively. These are further subdivided into proton peaks for specific groups. Any peak with known amount of protons in the spectrum can be utilised to calculate protons for other peaks in the spectrum. Any incomplete structure however, will change the ratio of protons between groups. The dendrimer structure is such that a correlation exists between any uncoupled lipidic chains and H_N protons. One uncoupled lipidic chain will result in 1 added proton on the external amino groups of lysine.

The integral of the peaks in the H_N spectra accounts for 65 protons, 31 lysine with 62 NH groups and 1 glycine with 1 NH and 1 NH₂, which is the total amount of protons of all the N groups on the dendrimer. The numeric value accounting for 65 protons was calculated as 4.83, as shown in Table 2.3.

Table 2.3. Showing the peak type, field placement (ppm) in the spectrum and intensity of the HN peaks.

H _N peak	ppm	End ppm	Intensity
Doublet	8.0	7.9	1.00
Doublet	7.7	7.6	1.12
Triplet	7.5	7.4	1.15
Triplet	7.4	7.3	1.17
Singlet	7.1	7.0	0.10
Singlet	7.0	6.9	0.08
Singlet	6.9	6.8	0.06
Total intensity			4.83

However, because the H_N protons were used as a reference, this value increased 1 H with each uncoupled lipidic chain. Using the peak of CH_3 as a indicator for the amount of lipidic chains (one CH_3 group for each lipidic chain), the following equation could be made (1):

$$\left(\frac{\int CH_3 peak}{\int H_N} \times (H_N \text{ protons} + X) = (CH_3 \text{ protons}) \Rightarrow \right. \quad (1)$$

$$\left. \frac{CH_3 \text{ protons}}{3} = (\text{theoretically calculated } CH_3 \text{ groups}) - X \right.$$

The value obtained from the integrals ($\int CH_3$ and $\int H_N$) represents the intensity of the CH_3 peak and H_N peaks respectively, H_N protons represents a theoretical calculation of the total amount of protons attached to N groups, X = uncoupled CH_3 groups: each CH_3 group missing corresponds to a lipidic chain missing which results in X added proton on the N groups. The total theoretical number of lipidic chains are 32. Therefore the following equations can be derived. The integral for CH_3 : 5.67 (2), - $(CH_2)_7$: 22.75 (3) and $-CO-CH_2-$: 3.99 (3).

$$\left(\frac{\int CH_3 peak}{\int H_N} \times (H_N \text{ protons} + X) = 3(32 - X) \right. \quad (2)$$

$$\left(\frac{\int CH_2 peak}{\int H_N} \times (H_N \text{ protons} + X / 7) = 2(224 - X) \right. \quad (3)$$

$$\left(\frac{\int CH_2 peak}{\int H_N} \right) \times (H_N \text{ protons} + X) = 2(32 - X) \quad (4)$$

Using Eq (2) it was calculated that dendrimer II had 85 % (27 lipidic chains) of the exterior amino groups coupled to decanoic acid. Using eq (3) and (4) the same calculations were made for $-(CH_2)_7$: 22.75 and $-\text{CO}-CH_2-$: 3.99 respectively and showed that between 27 and 29 dodecanoic acid chains were coupled to the amino groups of the external lysine. The integral peaks for all the dendrimers are listed in Appendix 1 and the calculated percentage of lipidic chains attached to the external lysine are stated in Table 2.4.

From the theoretical molecular weight, the molecular weight of the dendrimers without X amount of lipidic chains can be calculated and used to explain the spectra obtained from mass spectrometry (Example given in Figure 2.13). Because of the hydrophobicity of the dendrimers it was difficult to obtain mass spectra of the dendrimers. The lipidic chains suppressed the signal and therefore the height of the signals in a spectrum should not be compared because other signals in the sample reflects impurities, which might be easily ionised, and therefore shows up as a comparatively more intensified peak. Therefore the peaks were broad and showed a range of molecular weights. Using the information obtained from the NMR, the peaks of the mass spectra were identified.

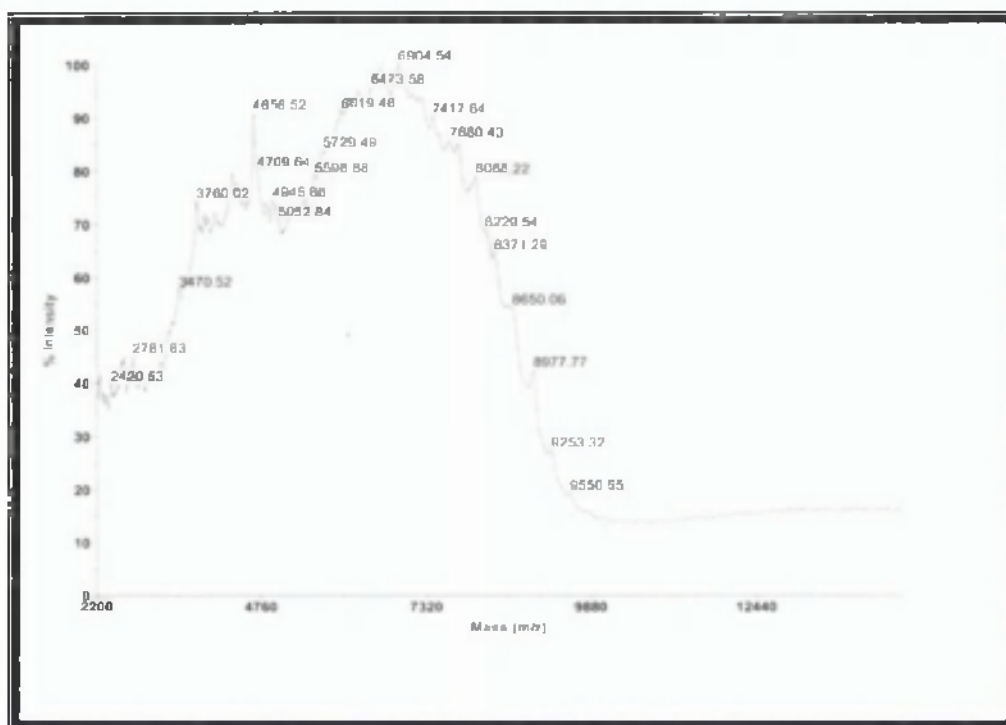


Figure 2.13. Mass spectrum of dendrimer II, $C_{508}H_{957}N_{64}O_{64}$ (8975.63): $8977 [M+2H]^+$ (40), $8371 [M-4 \times 155(C_{10}H_{19}O)+Na]^+$ (61), $8229 [M-5 \times 155(C_{10}H_{19}O)+Na]^+$

The mass spectrum confirms the molecular weight, an example of dendrimer II given in figure 2.13. However, the NMR and mass spectrum does suggest that fractions of dendrimer do exist, as dendrimers lacking lipidic chains were apparent in the NMR spectra. Table 2.4 states the theoretical molecular weight along with the practical values obtained. It could however be concluded, that at higher dendrimer generations, the coupling efficiency decreases. This may well be because of steric hindrance as an effect of the dense structure of the dendrimer. The NMR does show a large deviation from the molecular weight obtained from mass spectrometry. This might be because of the short scanning time, 3h, utilised when running the NMR. Mass spectrometry and NMR data are listed in Appendix 1.

Table 2.4. The theoretical molecular weights, the molecular weight determined by mass spectrometry and the molecular weight calculated from NMR. The results suggest that coupling efficiency decreases with the increase in dendrimer generation.

Dendrimer	Theoretical molecular weight, (Da)	Calculated molecular weight from mass spectrometry (Da)	Calculated molecular weight from NMR(Da)	Lipidic chains coupled to external amino groups (% of theoretical possible couplings)
I	6290.4	Exact	6077.1	91%
II	8975.6	Exact	8200.6	84%
III	9881.2	9680	8965.0	84%
VI	12590.8	Exact	10671.4	58%
V	18019.1	Exact	13834.0	57%
IV	19814.5	19081	15598.6	64%

2.4.5 Conclusion

A series of lysine based dendrimers were synthesised using Fmoc SPPS. The mass spectrometry confirmed the molecular weight of the dendrimers. It was, however, not possible to synthesise a dual label dendrimer, most likely because of detachment of the tritium label during the synthesis procedure. NMR suggested that the coupling efficiency of the dendrimer decreased as the dendrimer generation increased. The 5th and 6th generation dendrimers were ellipsoidal in shape and, because of their branched structure, provided an overall dense structural arrangement. The very low aqueous solubility of these dendrimers provided the opportunity to formulate the dendrimers into nanoparticles, i.e. systems containing multiple individual dendrimers. The diameter of these ranged from 5 to 50 nm. Their formulation is described and discussed further in chapter 3.

Formulation of dendrimer-derived nanoparticles

3.1 Introduction

It has been well documented, that physical characteristics including size and surface nature, affect nano- and micro- particulate translocation *in vivo*. Generally, the smaller the diameter the greater the extent of absorption; charged surfaces reduce particle absorption, while increasing hydrophobicity results in greater uptake and translocation (Florence 1997). However, several other factors are also of importance when using nanoparticles derived from lipidic subunits of individual dendrimers, such as described in the present chapter. Li *et al.* (2004) found the stability of dendrimer aggregates to be dependent on the structural flexibility of the individual dendrimer molecules. The nature of their packing into aggregates affects the size, shape, size distribution and colloidal stability of the formed particles and are factors which determine drug entrapment and the flow properties of dispersed particles.

Dendrimers are three dimensional hyper-branched macromolecules (Tomalia *et al.* 1990) with great potential as carriers because their surface properties can be modified to meet a variety of design criteria. Even though many structurally different dendrimers have been synthesised, the full potential of dendrimers as drug delivery vectors has yet to be fulfilled. If dendrimers are to be used as delivery systems it is important to understand the nature of packing during self-assembly (Emrick *et al.* 1999, Liu *et al.* 2000, Liu *et al.* 2003), formations of megamers (Tomalia *et al.* 2000, Fréchet 2002) and controlled aggregation with and without encapsulated drug molecules. Studies have been conducted on interactions of cationic dendrimers with DNA (Ramaswamy *et al.* 2003), complexation with or interaction of piroxicam (Wiwattanapatapee *et al.* 1999) and ibuprofen (Milhem *et al.* 2000), the formation of higher order structures (Al-Jamal *et al.* 2003) and the interaction of dendrimers with surfactants (Bakshi *et al.* 2004, Esumi *et al.* 2002).

The formulation of particles from microemulsions, using a variety of different polymers has been studied widely (Brown 2003). The potential of formulating larger particles by nanoprecipitation using dendrimers, is still to be explored. This chapter studies the formulation of dendrimer-derived nanoparticles from emulsions, and is a new and alternative method of assembling dendrimers.

3.2 Materials and Methods

3.2.1 Materials

Dendrimers I to VI were as described in chapter 2. Dichloromethane (DCM), ethanol, octanol and chloroform were purchased from BDH, Leicestershire, UK,

polyoxyethylene cetyl ether (EO, $n = 10$, MW 683) Emalex 110 from Nihon Emulsion Co Ltd, Japan and pyrene (99.9 %) from Lancaster, Morecambe, UK. OptiPhase 'Safe' scintillation fluid was purchased from Perkin Elmer LifeSciences, Loughborough, UK. Polystyrene nanoparticles ($214\text{nm} \pm 5\text{nm}$) used to calibrate the particle analyser (4700C sub-micron particle analyser Malvern instruments, Malvern, UK) was purchased from Malvern, UK. Wipes without fibers (Nima, Nima Technology Ltd, Coventry, UK).

3.2.1 Formulation of dendrimer-derived nanoparticles

The dendrimers, with molecular weights ranging from ~ 6200 to 19800 , differed in generation and length of lipidic chain (Table 1). Controlled aggregates of the dendrimers (I-VI) were formed using a precipitation method as described by Quintanar-Guerrero *et al.* (1998). Each dendrimer (I to VI) was solubilised in dichloromethane (DCM) and double distilled deionised water added. The solution was probe sonicated and the emulsion formed was stirred until the DCM evaporated and the dendrimer aggregates precipitated. The exact method used for the experiments are listed below and referred to in the text where necessary.

Method 1: Dendrimer III (0.25 mg) was solubilised in 1 ml DCM. Aliquots of double distilled deionised water was then added and the solution probe sonicated (2 x 30 s) and the emulsion formed stirred until all the DCM was evaporated.

Method 2: As described in method 1, but using 0.25ml DCM instead of 1 ml.

Method 3: As described in method 2, but using dendrimers I to VI

Method 4: As described in method 2, but using dendrimers I to III and only 30s probe sonication

Method 5: As described in method 2, but using dendrimers I to III. The surfactant (polyoxyethylene cetyl ether E10, $\text{CH}_3(\text{CH}_2)_{15}(\text{OCH}_2\text{CH}_2)_{10}\text{OH}$) was added to the water phase, to investigate the stability of the aggregates, in concentrations ranging from 0.5% w/w to 5% w/w.

3.2.2 Characterisation of dendrimer-derived nanoparticles

3.2.2.1 Particle diameter determination

Particle suspensions were sized by Photon Correlation Spectroscopy (PCS) using a Malvern 4700C sub-micron particle analyser (Malvern instruments, Malvern, UK). PCS measures Brownian motion and relates this to the size of the particles. Brownian motion is the random movement of particles due to bombardment by the solvent molecules that surround them.

The size of a particle is calculated from the diffusion coefficient using the Stokes-Einstein equation;

$$d_H = kT/3\pi\eta D$$

Where

d_H = hydrodynamic diameter

D = diffusion coefficient

k = Boltzmann's constant

T = absolute temperature

η = viscosity of the medium

The diameter, which is measured in PCS, is a value that refers to how a particle diffuses within a fluid and so it is referred to as the hydrodynamic diameter. The particle analyser measures the samples at a single angle, 90°. The upper limit for the size measurement is dependent on the density of the sample, and is determined by the onset of sedimentation in the sample. The lower limit is ~ 2nm which depends on the sample concentration (Malvern Zetasizer Manual, Malvern Instruments, Malvern, UK).

Table 3.1. The correlation between size, number and mass distributions (Rawle 2004).

Size (cm)	Number of objects	% by Number	% by Mass
10-1000	7000	0.2	99.96
1-10	17500	0.5	0.03
0.1-1.0	3500000	99.3	0.01
Total	3524500	100.00	100.00

The hydrodynamic diameter calculated by the particle analyser generates a volume distribution for the analysed light energy data. From this data the number average is calculated. Table 3.1 shows the correlation between size, number and mass distribution. The example shows the number and mass distribution of objects ranging from 1000 cm to 0.1 cm in diameter. The experiment was conducted to assess if a space shuttle needed protection against mainly small (<1 cm) or large (>1 cm) objects. The data clearly showed that if the data was interpreted by mass the larger objects had most significance and when interpreted by number values, the small objects were more significant. Number and mass distributions are very different from each other and different conclusions can be drawn from each distribution. Hence,

while interpreting results, one therefore has to consider which distribution is relevant. When studying oral uptake and translocation of particles, the smaller particles (<100nm) seems to be more significant as only they are translocated through to the systemic circulation. Where relevant in this thesis, number values have been quoted.

Experimental

The nanoparticle suspension size determination was measured using Acryl cuvettes (10 x 10 x 48 mm, Sarstedt, Germany) with four clear sides, all samples measured containing a volume of ~ 1 ml. Each preparation was analysed in triplicate using CONTIN to give an average value for particle diameter. The polydispersity was referred, indicating the particle size distribution of the measured sample.

3.2.2.2 Zeta potential measurement

The zeta potential of the suspension of dendrimer-derived nanoparticles was measured to study the particle interaction. Zeta potential it is the controlling parameter for particle interaction, and is dependent on the STERN layer and not the surface potential. The zeta potential (mV) was measured with a Malvern Zetasizer 3000 (Malvern Instruments, Malvern, UK), which employs the technique of Laser Doppler Velocimetry (LDV). The charge of the nanoparticles is determined by measurement of the migration rate of dispersed particles under the influence of an electric field and is given by the relationship:

$$\mu_E = V / E$$

Where μ_E is the electrophoretic mobility, V observed velocity and E is the electrical field.

The zeta potential (ξ) can be calculated from the mobility using Henry's equation

$$\mu_E = \varepsilon * \xi * f(\kappa a) / 6 \pi \eta$$

ε = dielectric constant of the medium

ξ = zeta potential

η = viscosity of the medium

$f(\kappa a)$ = correction factor, which takes into account the thickness of the double layer and the particle diameter.

Experimental

The zeta potential is obtained by dispersing 1ml of the dendrimer-derived nanoparticles (0.07 mg/ml to 1.71 mg/ml), derived from dendrimer III, in 9 ml of double distilled deionised water (MilliQ System, Millipore, USA). The 10 ml of the diluted sample was injected and analysed 3 times, giving an average value. Between measurement of each sample, the cell was washed with 50-100 ml of double distilled deionised water, to flush out any residual materials.

3.2.2.3 Transmission Electron Microscopy

Dendrimer-derived nanoparticles were produced as described in section 3.2.1. 2 μ l of the dendrimer-derived nanoparticle suspension was added on to the grid and stained with 1% uranyl acetate. The stain was filtered off and the procedure repeated. The grid was air dried and photomicrographs were recorded at magnifications ranging from 52,000 to 105,000 at an operating voltage of 120 kV.

3.2.2.4 Polarity measurements

The packing of the dendrimers in aggregates was investigated using pyrene as a hydrophobic fluorescent probe (excitation, 340nm) (Perkin Elmer, Spectrophotometer LS 50B, UK). The intensity, the peak height, ratios of the first band (374nm) to the third band (385nm) (I_1/I_3) in the emission spectra (350-600 nm) were determined as a function of dendrimer concentration. The changes in the vibronic peaks I_1 and I_3 is directly related to the polarity of the environment, a decrease in I_1/I_3 indicates an increase in the non-polar environment. Pyrene in ethanol solution (0.4 mM, 6 μ l) was added to the dendrimer suspension (0.09 mg/ml to 1.71 mg/ml) in a total volume of 3.5ml. The samples containing 0.69 μ M pyrene were kept for 24h at 20°C before measurements were made. A spectrum of saturated pyrene in water is shown in figure 3.1.

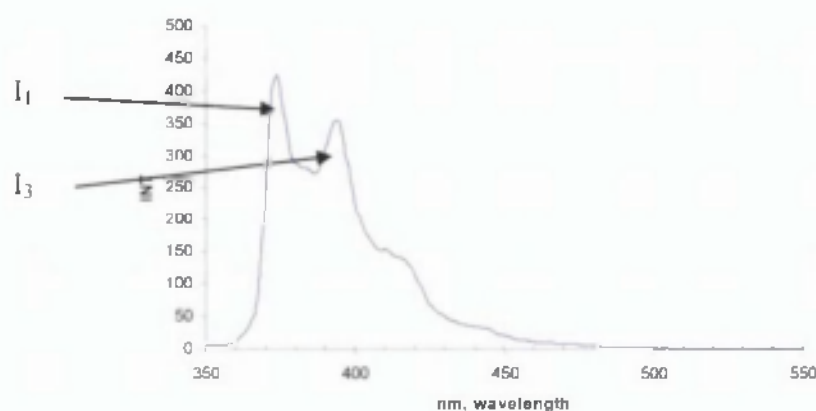


Figure 3.1. The spectrum of water saturated with pyrene. The figure shows the five vibronic peaks with two vibronic peaks marked on the graph, I_1 and I_3 .

2 ml of each sample was then measured using a Quartz cuvette and spectra obtained using a spectrophotometer (Perkin Elmer, LS 50 B, UK). The intensity ratios I_1/I_3 in the emission spectra were determined as a function of dendrimer concentration to study the changes in the polarity as the packing density increased.

3.2.2.5 Surface area measurements

Dendrimers were studied at the air-water interface to gain better understanding of the packing of dendrimer-derived nanoparticles. The surface area per molecule in spread monolayers of dendrimers was measured using a Langmuir trough (Nima, Nima Technology Ltd, Coventry, UK) (Figure 3.2). This had two movable teflon barriers and a Wilhelmy plate (Nima Technology Ltd, Coventry, UK) was utilised to determine surface tension, γ , and hence surface pressure π . In the Wilhelmy plate technique, the vertical force acting on the plate, which is partially dipped in the subphase is measured. The forces acting upon the plate being gravity and surface tension. Surface tension expresses the energy per unit area while pressure expresses the energy per unit volume. Surface tension in any liquid is created because liquids always try to reduce their interfacial area. Surface pressure is simply another way of expressing the lowering of surface tension caused by a surface film. Compared to a clean water surface, a monolayer on the water surface lowers the surface tension and can be used to determine the lowering of the surface pressure, π :

$$\pi = \gamma_0 - \gamma$$

where γ_0 is the interfacial tension of the clean water surface and γ is the interfacial tension with the monolayer present. The surface pressure, π , is the negative of the change in surface tension.

The π/A -isotherm (Figure 3.3) is obtained by measuring the surface pressure, π , during the monolayer compression by decreasing the area of the monolayer with the movable Teflon barriers. The various phases of the π/A -isotherm resemble the two-

dimensional analogues of gases, liquid and solids. From the π/A -isotherm the area per molecule can be extrapolated (A_0) and the shape of the isotherm can be used to determine the nature of the monolayer and its degree of packing (Shaw 1980, Roberts 1984, Florence and Atwood 1998).



Figure 3.2. The Langmuir Blodgett trough was utilised in the experiments. The trough has two mechanical removable Teflon barriers. The pressure sensor was mounted on the edge of the trough and used a Wilhelmy plate to measure the surface pressure (reduction in surface tension). The volume of the trough was 0.50 cm x 13.5 cm and 8 cm. Tubing connectors was available for temperature control.

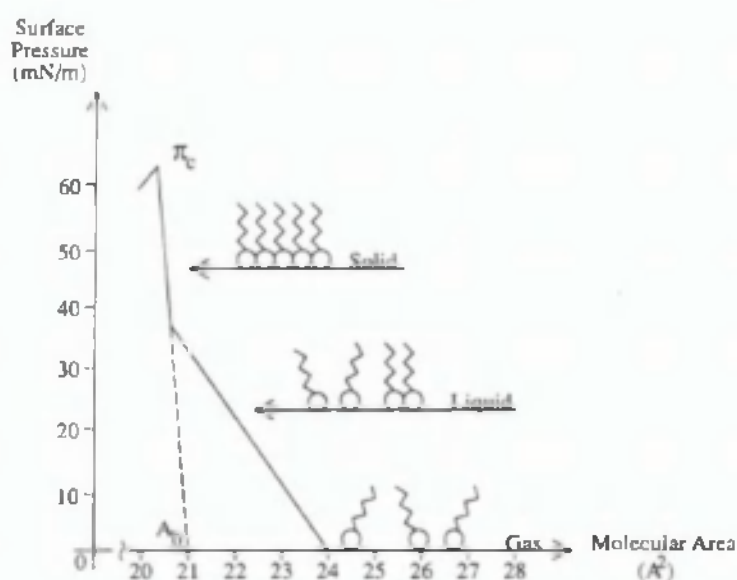


Figure 3.3. A π/A -isotherm of stearic acid on distilled water. Various phases of the π/A -isotherm are shown; gas expanded to condensed liquid to two dimensional solid phase (Martin and Szabewski 2002).

Experimental

Dendrimers I to VI, as described in chapter 2, were dissolved in DCM (1mg/ml). The solution was added dropwise to the surface of the double distilled deionised water. The total volume added onto the trough was recorded. The compression rate was 10 cm²/min and the temperature was kept constant at 25°C. The trough was cleaned with chloroform before and after each experiment and wiped clean with wipes without fibers supplied by Nima.

3.3 Results and discussion

3.3.1 Formulation of dendrimer-derived nanoparticles

The series of dendrimers, described in chapter 2, were synthesised to study their biodistribution following oral administration. Ideally an aqueous formulation would be preferred, as most organic solvents would not be appropriate to administer because of potentially severe toxic side effects. Because dendrimers I to VI were all insoluble in water, a controlled assembly of individual dendrimers into larger aggregates was formulated using a precipitation method as employed by Quintanar-Guerrero *et al.* (1998).

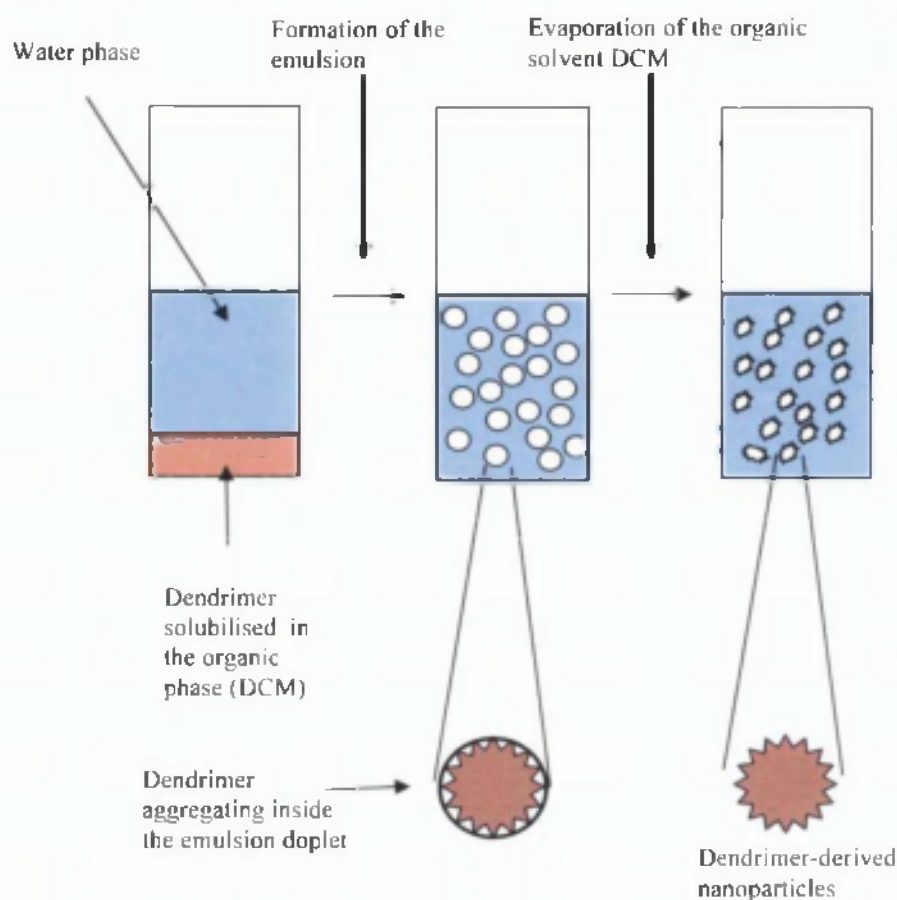


Figure 3.4. Schematic representation of the precipitation method used for the formulation of controlled dendrimer-derived aggregates. Dendrimers were solubilised in DCM and water was added. The samples were probe sonicated to form an emulsion and stirred until all DCM was evaporated. As the DCM evaporated the dendrimer-derived nanoparticles precipitated out to form a aqueous suspension.

As described in section 3.2, the dendrimer-derived particles were formulated using method 1. The solution was sonicated for 2x 30s, and the emulsion formed was stirred until the DCM was evaporated and the dendrimer aggregates precipitated out to form an aqueous suspension (Figure 3.4). Initially, the effect of different volume ratios between the dispersed phase and the dispersion medium at different dendrimer (III) concentrations (0.014% w/v and 0.1% w/v) was assessed (Table 3.2). By changing the ratio between the internal and the external phase it was possible to

control the Z-average of the particles. Table 3.2 shows the resulting effect at two different dendrimer concentrations (0.014% w/v and 0.1%w/v).

Table 3.2. The effect of ratios between DCM/water of the emulsion at two different dendrimer concentrations on the Z-average of the dendrimer-derived nanoparticles.

Dendrimer III concentration in DCM	ratio DCM/water (total volume 4ml)	Z-average diameter +/- stdev, nm	Polydispersity index
0.014% w/v	1:1	1111 $\pm$ 239	0.80
	1:3	397 $\pm$ 26	0.45
	1:6	285 $\pm$ 14	0.51
0.1% w/v	1:3	480 $\pm$ 12	0.30
	1:10	161 $\pm$ 5	0.40
	1:12	165 $\pm$ 4	0.31
	1:14	168 $\pm$ 4	0.35

This table shows that as the DCM/water ratio decreases, the Z-average diameter of the resulting dendrimer-derived nanoparticles decreases (n=3).

At the lower dendrimer concentration a decrease in the Z-average diameter (~1100nm to 300nm) of the dendrimer-derived nanoparticles was observed, as the volume of the dispersion medium was decreased, corresponding to a ratio of 1 to 0.17 between the internal/external phase.

The increase in dendrimer (III) concentration from 0.014% w/v to 0.1% w/v seemed to stabilize the formed droplets, resulting in lower polydispersity of the dendrimer-derived nanoparticles. Changing the DCM/water (total volume 4 ml) ratios from 0.33 to 0.07 resulted in a decrease in Z-average of the dendrimer-derived nanoparticles (from 480nm to 168nm). This suggested that the droplet formed by the emulsion and the dendrimer concentration in the internal phase of the emulsion, *inter alia*, governs the diameter of the ultimate nanoparticles. In principle there are two different processes that provide instability of emulsions: 1) diffusion of the nonaqueous phase

(Shaw 1980 and Everett 1988). In a solution with insoluble substances Ostwald ripening is negligible, as diffusion from smaller to larger droplets mainly occurs if the smaller particles dissolve and therefore the larger particles grow on their expense. The main parameter, besides dendrimer (III) concentration and sonication time, which was kept constant, that determined the formation of dendrimer-derived nanoparticles, was therefore possibly the droplet coalescence. The coalescence of droplets did seem to have an effect on the mean number diameter of the ultimate particle formed (see section 3.3.1.1) and was kept to a minimum because of the very short evaporation time of DCM.

In a recent article, Rosca *et al.* (2004) studied the formation of microparticles from an O/W emulsion by following the evaporation of the solvent from emulsions of 1% w/v PLGA and 1% w/v PLA on a cover glass. They showed how the solvent of the droplets diffused through the aqueous phase resulting in the microparticles precipitating out into the water on the cover glass. Furthermore, a significant shrinkage of the resulting microparticles was observed compared to the initial droplet size. The shrinkage depends on polymer concentration and stirring rate and time. The interaction of dendrimers via the internal phase, after droplet formation, seems of secondary importance.

Measuring the overall charge of the dendrimer-derived nanoparticles using dendrimer (III) (0.07 mg/ml) at a ratio of 1:14 (o/w), showed an overall positive zeta potential ($\sim +12\text{mV}$). This explains the stability of the ultimate particle in an aqueous media. Having an overall positive surface potential makes the dendrimer-derived nanoparticles repel each other to create a stable suspension. The positive surface potential mainly exists because of the amine group on the glycine in the core of the

single dendrimer molecule. However, free NH_2 groups present because of insufficient coupling of the lipidic chain to the surface amino groups, as suggested by the NMR data, chapter 2, also contributed to the surface potential (84 % coupling efficiency). Aggregation of the dendrimers into dendrimer-derived nanoparticles therefore also showed an over all charge. Furthermore, the nanoparticles (ratio of 1:14) have a pH of ~ 6 in suspension, contributing to a positive charge of the amino groups.

By further changing the formulation parameters such as the dendrimer generation, lipophilicity and dendrimer concentration, the particle diameter and hydrophobic nature of the aggregates was investigated. The data gained from π -A studies (section 3.3.3) at the air/water interface have allowed some theoretical calculations on the packing of the dendrimers in their nanoparticles.

3.3.2 Effect of dendrimer generation on apparent particle diameter

Using the same amount of dendrimer (0.071 mg/ml), the same volume (0.25 ml DCM) of the organic phase and a sonication time of 2x30s (method 3), the effect on the Z-average and number average diameter (nm) of batches of particles prepared from dendrimers I to VI was assessed (Figure 3.5).

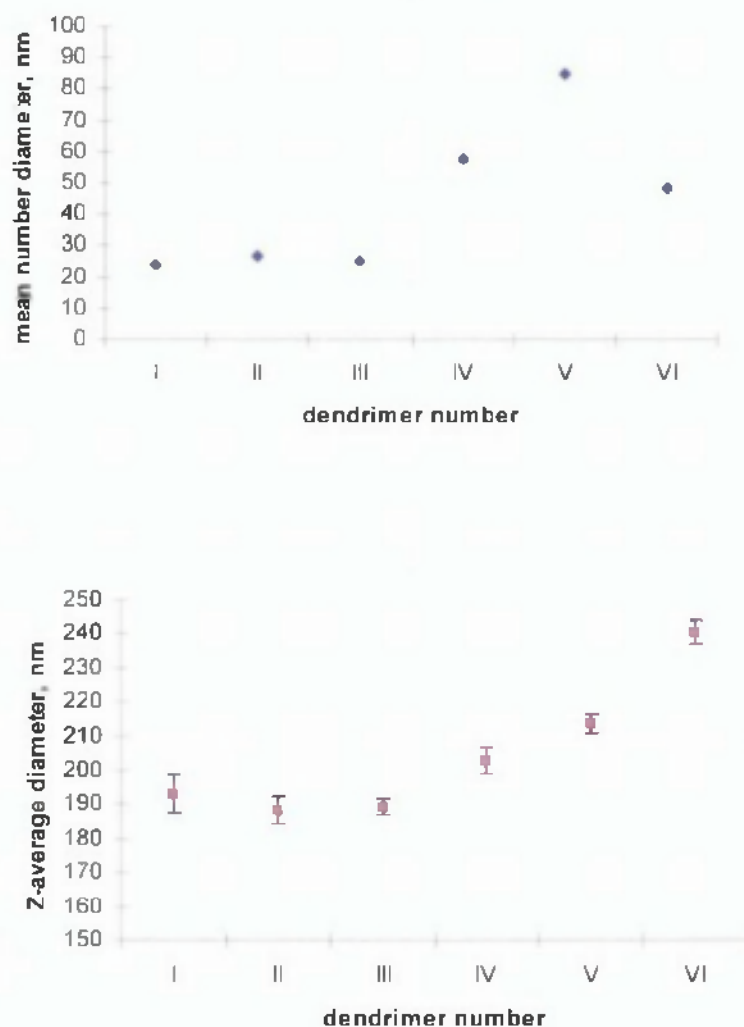


Figure 3.5. Effect on apparent particle diameter (nm) of batches prepared from dendrimers I→VI. I, II and III are 5th generation dendrimers with surface chains C₄, C₁₀, C₁₂ while IV, V and VI represents the 6th generation dendrimers with C₄, C₁₀, C₁₂. The concentration of dendrimers was 0.72mg/ml. Comparing 5th and 6th generation of dendrimers: I (192nm ± 6nm) with IV (204nm ± 3.8nm), II (189nm ± 4nm) with V (213nm ± 3nm) and III (190nm ± 2nm) with VI (240nm ± 3.7nm) an increase in Z-average (lower graph, n=3) and mean number values (upper graph) of the dendrimer-derived nanoparticles was observed. An increase in the surface lipid lipidic chain length from C₄ to C₁₂ had no effect on the particle diameter of aggregates derived from 5th generation dendrimers, and a small and variable effect on the 6th generation derived nanoparticles. Using similar molar ratios extrapolated from figure 3 did not alter the graph significantly.

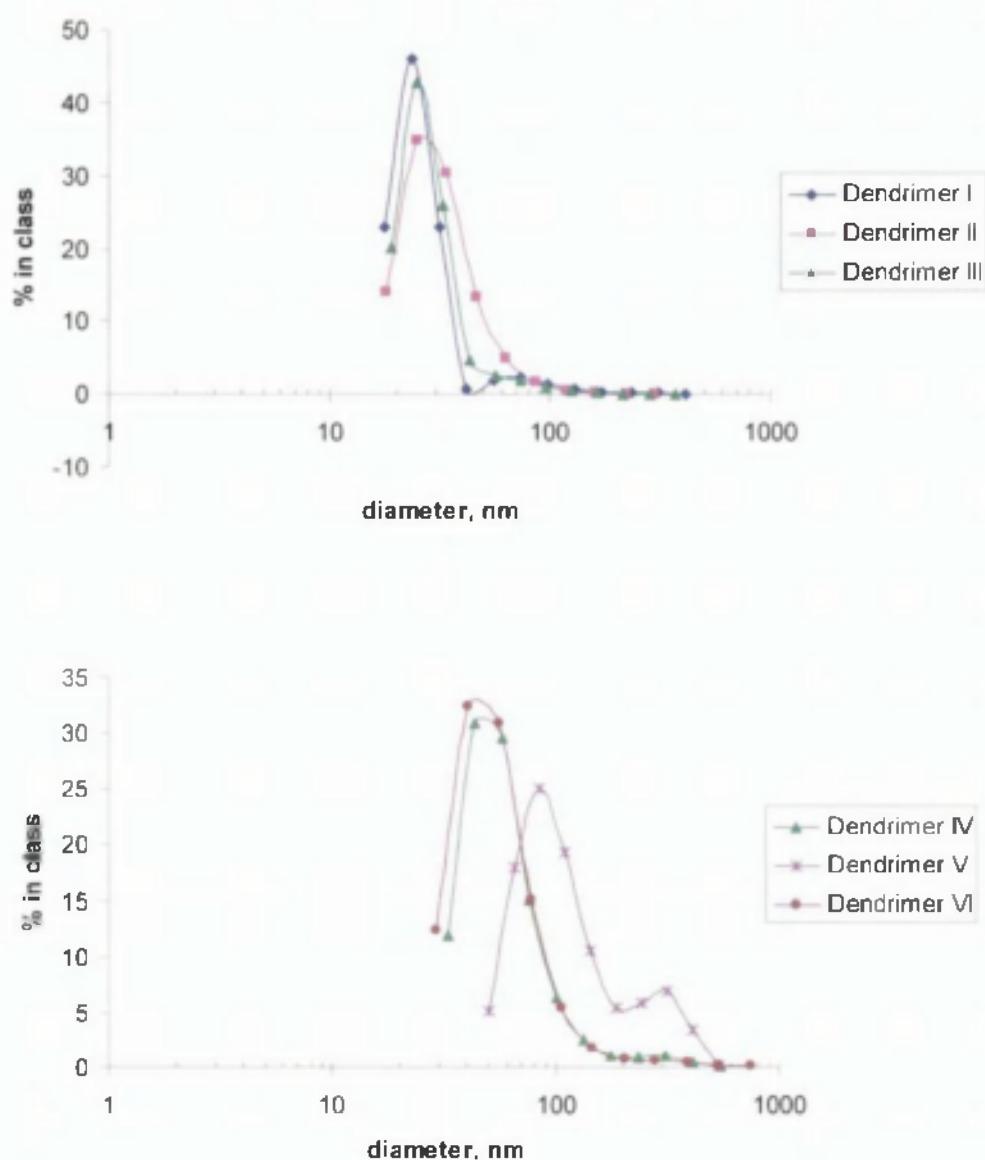


Figure 3.6. Showing the % in class of the mean number size distributions of the 5th generation dendrimer derived nanoparticles (upper graph) and 6th generation dendrimer derived nanoparticles (lower graph). Nanoparticles derived from the 6th generation dendrimers form larger particles than nanoparticles derived from the 5th generation dendrimers and tend to form a higher percentage of larger aggregates.

Compounds I, II and III denotes dendrimer-derived nanoparticles, derived from the 5th generation dendrimers with surface chains C₄, C₁₀ and C₁₂ respectively while IV, V and VI represent dendrimer-derived nanoparticles derived from the 6th generation dendrimers with C₄, C₁₀ and C₁₂ surface chains respectively. A slight increase in the Z-average diameter was observed when comparing 5th generation dendrimer-derived

nanoparticles with 6th generation dendrimer-derived nanoparticles: I with IV, II with V and III with VI. A similar trend was found when comparing the mean number diameters of the particles derived from the 5th and 6th generation dendrimers: I (26nm) with IV (57nm), II (27nm) with V (85nm) and III (25nm) with VI (48nm). More than 98.5 % of all particles derived from the 5th generation dendrimers were less than 100nm in diameter, whereas particles derived from the 6th generation dendrimers IV and VI had around 90%, and dendrimer V around 70% of all particles less than 100nm (Figure 3.6). This suggests that changing the dendrimer from 5th to 6th generation increased the number of larger aggregates. The increase in the number of lipidic chains on the dendrimers, from 32 to 64, is logically the cause of this increased aggregation.

3.3.2.1 Effect of lipidic chain on apparent particle diameter

Increase in the carbon chain length as in dendrimer I to III had no effect on the Z-average diameter of the nanoparticles (~190nm). Dendrimers IV to VI form particles with measured diameters from 204nm $\pm$ 3.8nm to a maximum of 240nm $\pm$ 3.7nm when the lipidic chain was increased (C₄ to C₁₂). The increase in Z-average diameter of particles derived from dendrimer IV to VI was caused by the aggregation and hence polydispersity of the nanoparticles. The 6th generation dendrimer with C₁₀ lipidic chains (dendrimer V) had fewer particles (~ 70 %) under 100nm and a larger mean number diameter (85nm) than the other members of the same generation. This trend was not observed for the 5th generation dendrimers. Only when the number of lipidic chains on the dendrimer increased did the difference become more significant. How exactly the C₁₀ lipidic chain, compared to the C₄ and C₁₂, changed the formation of the aggregates is not clear. Transmission electron microscopy indicates

that the apparent particle diameter of the aggregates is in the range ~20-50 nm, similar to the mean number averages (Figure 3.7).

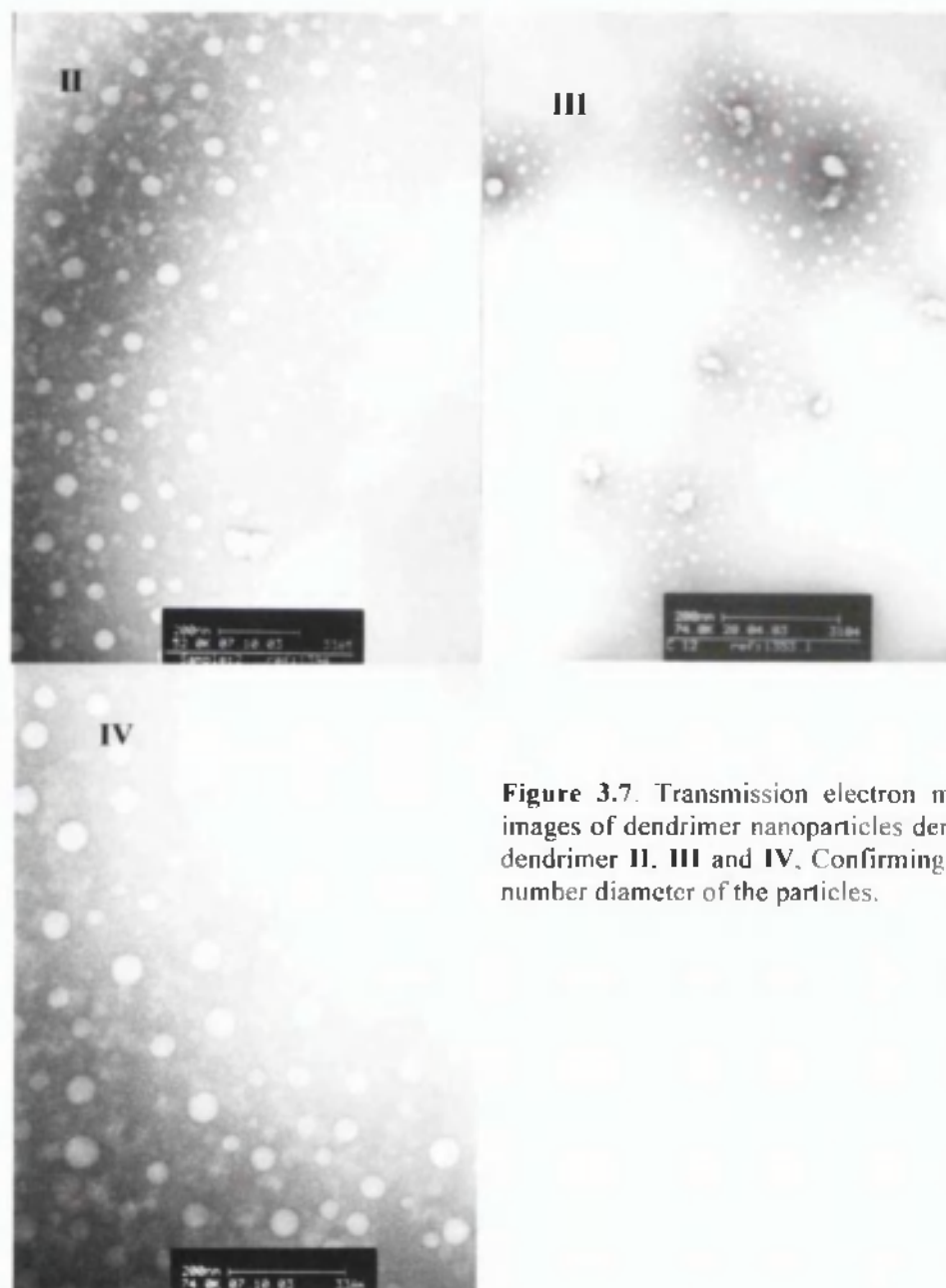


Figure 3.7. Transmission electron microscope images of dendrimer nanoparticles derived from dendrimer II, III and IV. Confirming the mean number diameter of the particles.

3.3.3 Surface area/molecule using langmuir trough

To gain a better understanding of the packing of dendrimer-derived nanoparticles, dendrimers were studied at molecular level. The surface area per molecule was measured as described. The π/A -isotherm for each dendrimer was recorded as described in section 3.2.2.5. An example of a π/A -isotherm from dendrimer I to III (Figure 3.8) and dendrimer IV to VI (Figure 3.9) are shown.

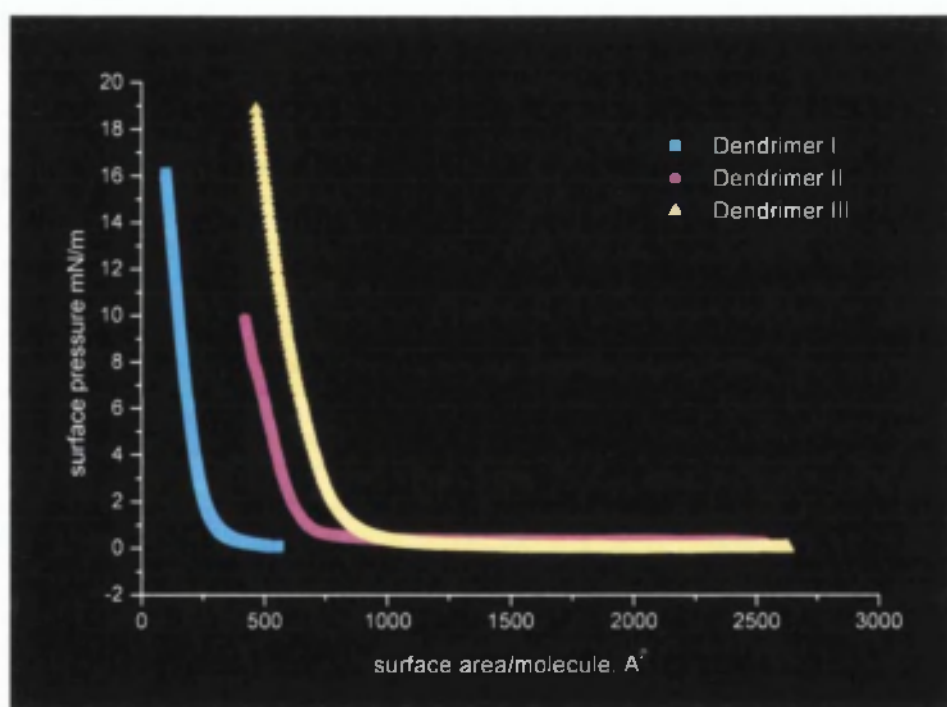


Figure 3.8. The π/A -isotherm of dendrimers I to III spread from DCM at the air/water interface, which shows an expanded monolayer. However, comparing the isotherm with the ones obtained from dendrimer IV to VI (Figure 3.9) they are at a more condensed state.

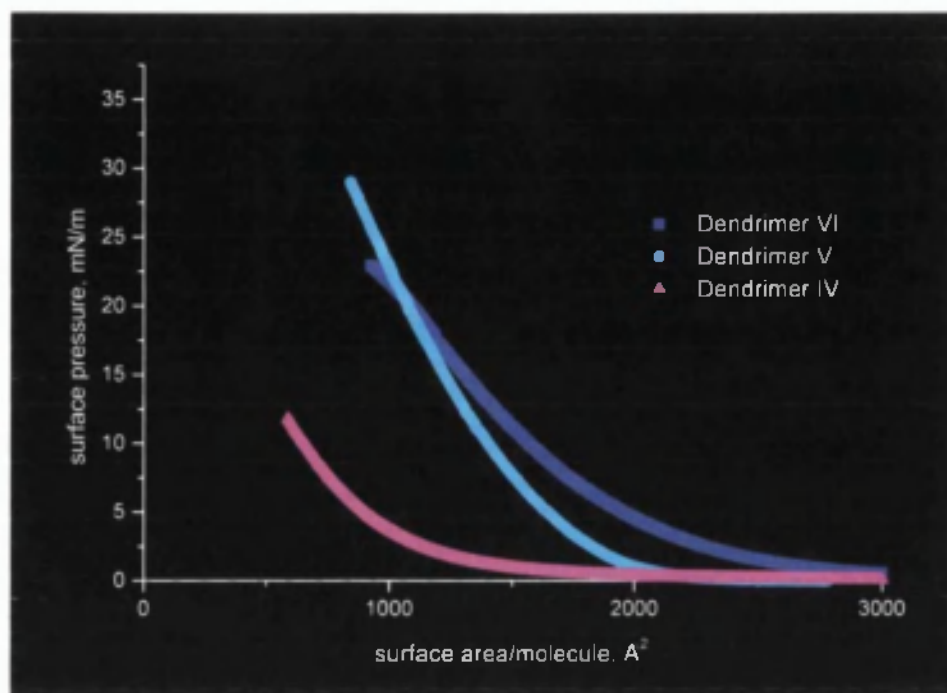


Figure 3.9. Showing the π/A -isotherm of dendrimer IV to VI spread from DCM at the air/water interface, which shows an expanded monolayer.

Various monolayer states exist (Florence & Attwood 1998). The isotherms formed by dendrimers I to VI are very similar to those observed for expanded monolayers. This represents an intermediate phase between gaseous and condensed film. Normally this type of film is formed by molecules where close packing is prevented (i.e. by bulky side chains). However, it has also been shown that latex particles behave in similar fashion at the air/water and oil/water interface (Marchenko *et al.* 2002). One reason for the formation of expanded films using hydrophobic dendrimers could be explained when considering the dendrimer hydrophobicity. Even though hydrophobic they exist as charged molecules (diffused electric double layer, DVLO theory) and therefore repel each other. Consequently, it is difficult to obtain a condensed monolayer. The larger the dendrimer, the greater the determined area per molecule. Shown in Figure 3.10, is an illustration of the possible monolayer

formation. Depending on the orientation and the contact angle of the dendrimer at the interface, the area per molecule changes. Furthermore, assuming that a dendrimer with more lipidic chains will have a smaller part in the subphase (contact angle more than 90°), the repulsion between the dendrimers will increase and create a more expanded monolayer because of the polarization of the counter ions.



Figure 3.10. Examples of two possible arrangements of dendrimers at the air/water interface. The upper figure show a larger amount of dendrimer in the subphase (contact angle less than 90°) and the lower a smaller amount ($\theta > 90^\circ$). Depending on the arrangement of the dendrimer in the subphase, repulsion between them will increase, *inter alia*, the surface area per molecule increases.

From the isotherms the surface area per molecule can be extrapolated as shown in Figure 3.11.

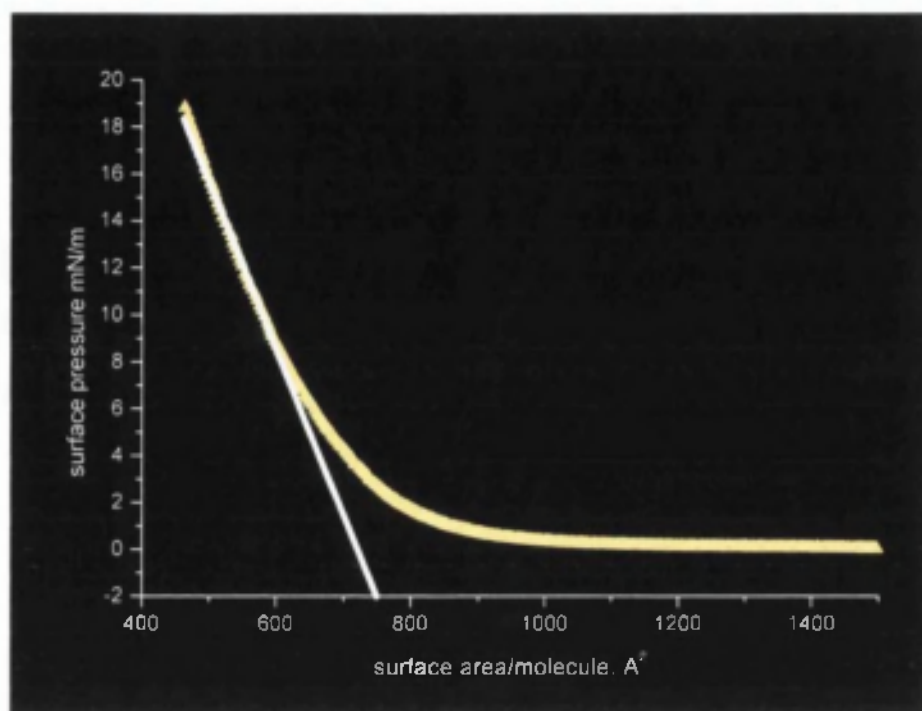


Figure 3.11. The extrapolation of surface area/molecule from the π/A -isotherm obtained from dendrimer III.

The extrapolated values are shown in Table 3.3. Also shown in the table is the calculated radii from the cross section area, equation 1, and the volume used for each dendrimer.

$$(1) \quad A = \pi r^2$$

Table 3.3. The area/molecule (nm^2) and radii (nm) for dendrimers I to VI spread monolayers.

Dendrimer	I	II	III	VI	V	IV
Area/ molecule ($\text{nm}^2 \pm \text{stdev}$)	2.49 ± 0.2	6.9 ± 0.3	7.8 ± 0.3	11 ± 0.1	21 ± 1.5	26 ± 2.8
radii (nm)*	0.9	1.5	1.6	1.9	2.6	2.9

* Derived from the average area/molecule ($n=4$).

The calculated radius for each dendrimer was then compared with values obtained from molecular models, Chapter 2, section 2.4 (Table 3.4). The dendrimers appeared to be more ellipsoidal than spherical in vacuum. Table 3.4 shows the measured diameters for each dendrimer and the resulting cross sectional areas, a and b. The cross sectional area per molecule was then compared with the data obtained from the Langmuir isotherms. The values shown in Table 3.5 are experimental areas per molecule divided by the theoretical areas per molecule. As mentioned above, the dendrimers seemed to be ellipsis (slightly flattened and elongated). Therefore two comparisons have been made, either assuming diameter, a, or diameter, b. Using diameter, a, the comparison showed a ratio of ~ 0.50 to ~ 0.60 between experimental and theoretical area per molecule of dendrimer I to III, suggesting that the 5th generation dendrimers are arranged at the air-water interface with the bulk of the molecule in the subphase, Figure 3.12a. The 6th generation dendrimers showed a ratio of 1.04 to 1.12, indicating a small part in the subphase and a expanded monolayer, Figure 3.12bc. Diameter, b, showed a ratio of 0.14 to 0.64 of dendrimers I to VI, suggestive of a monolayer largely subsiding in the subphase. From the data it seems more likely that the monolayer formation is with the dendrimers arranged with diameter, a, horizontal at the air/water interface (Figure 3.12). Mainly because the data from Table 3.5, diameter a, correlates with the Langmuir isotherms. The larger the dendrimers the more expanded the monolayer isotherms. Normally an expanded monolayer results in a cross section area higher than the actual diameter of the

molecule which is only apparent if the formation at the water interface is expanded as in Figure 3.12 b,c.

Table 3.4. The theoretical diameters and cross sectional area of dendrimers I to VI from molecular modeling.

Dendrimer	Diameter, a (nm)	Cross section area calculated from diameter a, nm ²	Diameter, b (nm)	Cross section area calculated from diameter b, nm ²
I	2.5	4.9	4.7	17.4
II	3.8	11.3	6.0	28.3
III	4.2	13.9	6.6	34.2
VI	3.7	10.6	5.5	23.8
V	5.0	19.6	6.7	35.3
IV	5.6	24.6	7.2	40.7

Using three dimensional molecular modelling, the diameters were estimated, by measuring the distance from one side to another using a straight line

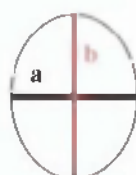


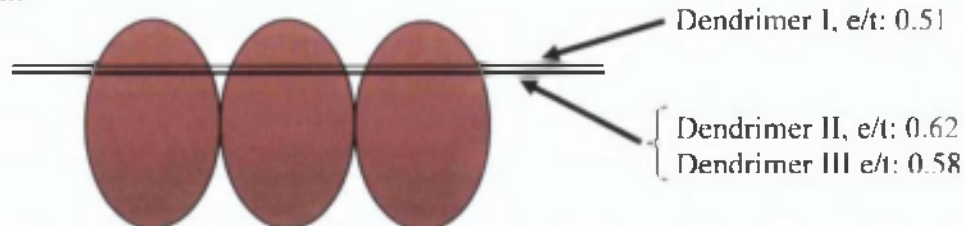
Table 3.5. Ratio between experimental and theoretical area/molecule.

Dendrimer	I	II	III	VI	V	IV
(Area per molecule)/(Area per molecule, a)	0.51	0.62	0.58	1.04	1.12	1.06
(Area per molecule)/(Area per molecule, b)	0.14	0.25	0.23	0.46	0.62	0.64

a and b represents the two diameters of the ellipsis.

a)

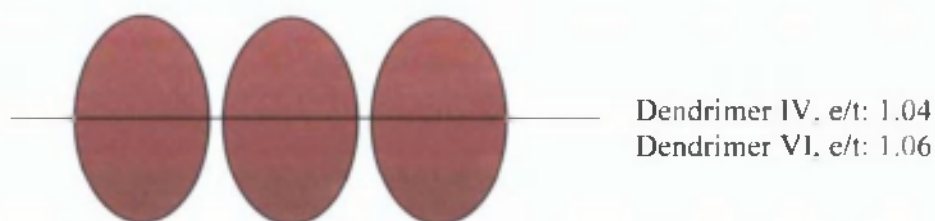
Air



Water

b)

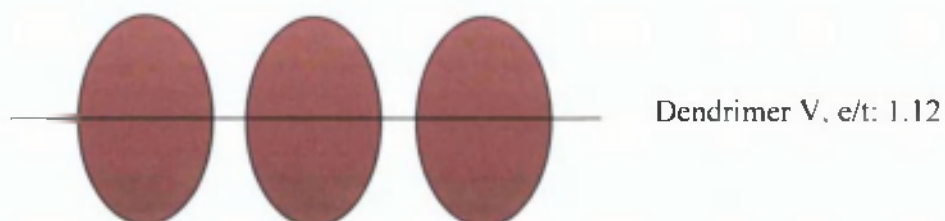
Air



Water

c)

Air



Water

Figure 3.12. Diagrammatic representation of the orientation of the dendrimers at the air/water interface and the depth of the dendrimers in the subphase, represented by the ratios between experimental/theoretical (e/t) values. a) showing dendrimer I, II and III forming a relative condensed monolayer with a larger part in the subphase. b) dendrimer IV and VI showing a slightly expanded monolayer and c) showing an expanded monolayer of dendrimer V.

The data suggests that dendrimers of the 5th generation forms more condensed monolayers than the 6th generation dendrimers. As the generation increases the monolayer the dendrimers form become more expanded. For both generations of dendrimers the trend was the same for the experimental/theoretical cross section area; $C_4 < C_{12} < C_{10}$. As the lipidic chain increases the cross section area should increase, however, the cross section area for dendrimers with C_{12} lipidic chains decreases, suggesting that flexibility of the lipidic chains affect the system.

The experimental/theoretical cross section area ratios correlates with the data from section 3.3.2, which showed that dendrimer II and V formed larger particles than the other dendrimers in their generation. The ratios similarly showed more expanded monolayers at the air/water interface for dendrimer II and V then other dendrimer in their generation. This suggested that dendrimer with C_{10} lipidic chains on their surfaces exists in a more expanded form and therefore formed larger dendrimer-derived nanoparticles. As the carbon chain length increases the ratio decreases again which could be due to the flexibility of the carbon chain.

3.3.3 Effect of dendrimer concentration on apparent particle diameter and zeta potential

To further study the packing of the dendrimers, the effect of dendrimer concentration on particle diameter, zeta potential and the hydrophobicity of their aggregates (using pyrene as hydrophobic fluorescent probe) was also studied. Particles were formulated as described in section 3.3.1, method 1. The sonication time, however, was changed to 30s. This change was made to emphasise any correlation between sonication time, droplet size, dendrimer concentration and the packing of the dendrimer-derived

nanoparticles. Particles of dendrimer I showed an increase in mean number diameter from ~30nm to ~50nm, which represents a 462% increase in volume, as the dendrimer concentration increased from 0.07 mg/ml to 1 mg/ml whereas dendrimers II and III showed a slight decrease in Z-average diameter and mean number diameter over the concentration range 0.07mg/ml-0.45mg/ml (Figure 3.13). The increase in amount of small particles in the systems (<100nm) led to an decrease in particle diameter (Figure 3.14). With increasing dendrimer concentration, a decrease in the diameter of particles derived from dendrimer II and III resulted in a increase of the zeta potential. The results, showed an increase in the zeta potential (from +23mV to +43mV) of dendrimer-derived nanoparticles (dendrimer III) as the concentration increased (0.07 mg/ml to 1 mg/ml)(Figure 3.15). The zeta potential increased rapidly at the lower dendrimer concentrations to reach a plateau. Because the particle diameter decreased slightly as the dendrimer concentration increased, the initial raise in zeta potential suggests an increase in density of dendrimer-derived nanoparticles. The plateau suggests that a compact nature of the particles were reached and also that further aggregation of the dendrimer-derived nanoparticles was minimal.

These results suggest, that dendrimers with surface lipidic chains (C_{10} to C_{12}) are likely to form condensed aggregates as the dendrimer concentration increased, leading to a smaller (dendrimer II and III) particle diameter. Increasing the dendrimer (II and III) concentration physically pushed the dendrimers together and encouraged hydrophobic interaction. This effect was not observed for dendrimer I with surface lipidic chains (C_4). Butyric acid has a length of ~0.6nm, significantly shorter than C_{10} and C_{12} lipidic chains, 1.2 nm and 1.6 nm respectively, which results in a more 'ridged' dendrimer with less space of interaction and therefore a decrease in particle diameter was not observed. The packing seemed mainly governed by hydrophobic

interactions between the lipidic chains on the dendrimer surfaces. However, the flexibility of the surface structures of the dendrimers on packing might be the reason that the dendrimer with the C₄ lipidic chains behaves differently from the dendrimers with longer lipidic chains (dendrimer II and III).

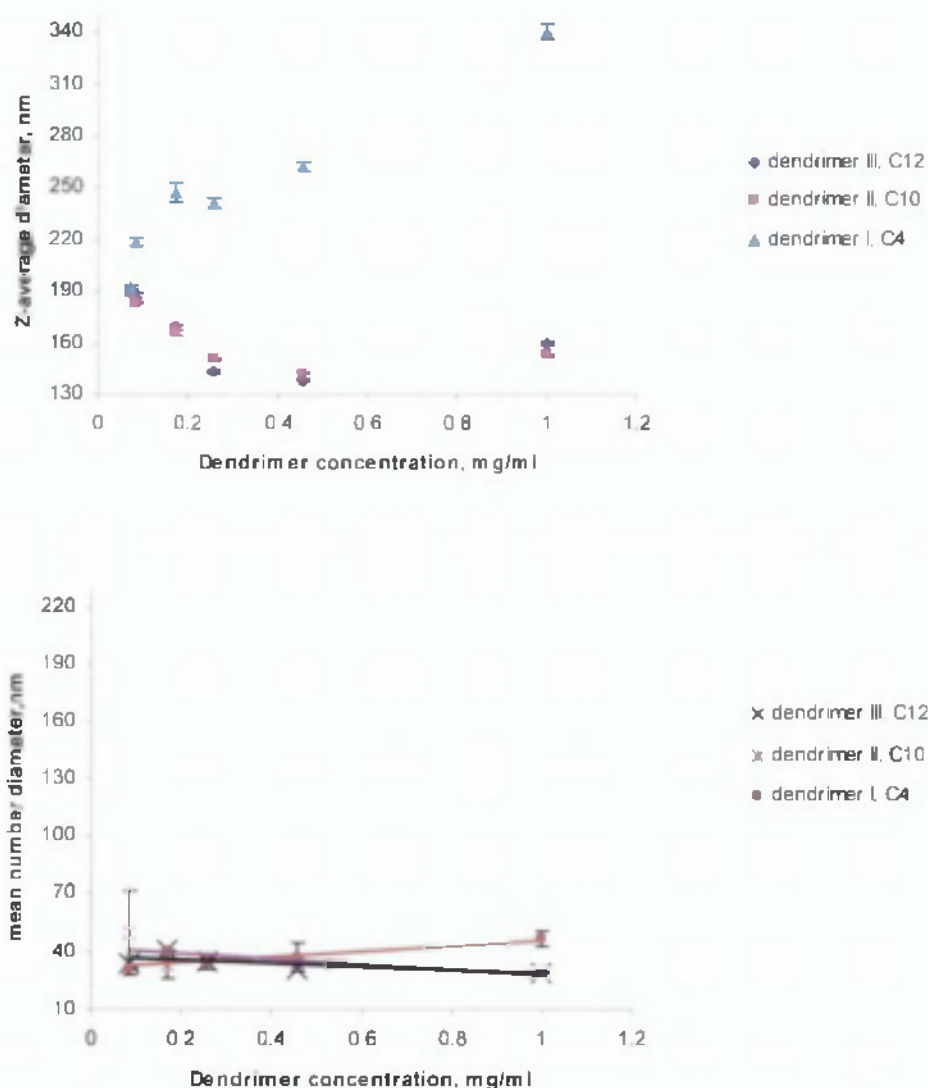


Figure 3.13. Effect of dendrimer (I, II and III) concentration (mg/ml) on particle diameter (Z-average and mean number diameter)(nm) of the aggregates using PCS. Increasing the concentration of dendrimer II and III resulted in a decrease in Z-average (~190nm to ~160nm and mean number diameter (~40nm to ~20nm). Dendrimer I, however, showed an increase in Z-average (192nm to 339nm) and mean number diameter (~30nm to ~50nm) as the concentration of dendrimer increased (n=3).

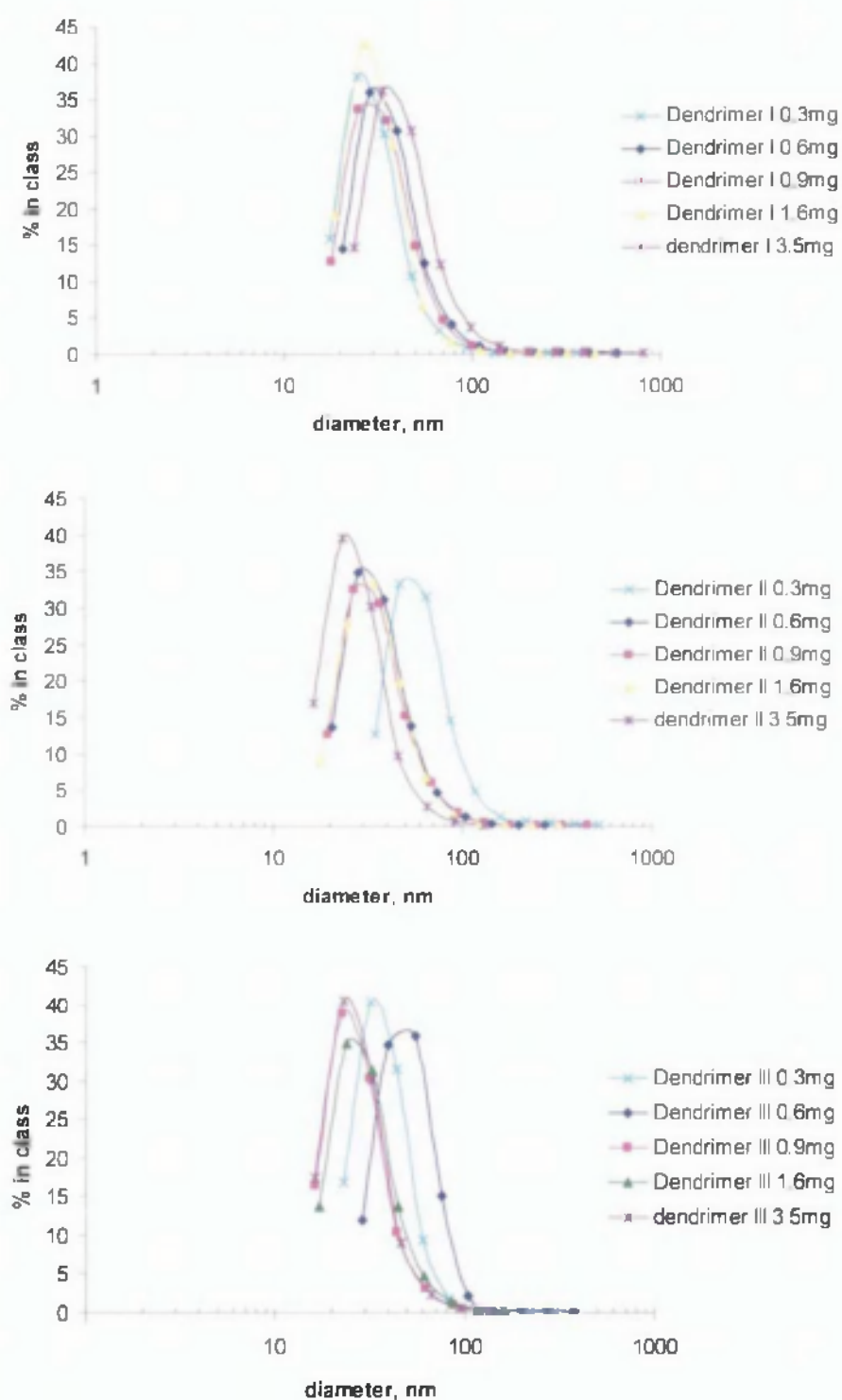


Figure 3.14. Diagram showing the percentage of particles in class in mean number size distributions of the 5th generation dendrimers **I** (upper graph) **II** (centre graph) and **III** (lower graph). Increasing the dendrimer concentration increased the amount of small particles in the system (<100nm) and lead to a decrease in mean number diameter for dendrimers **II** and **III**. The opposite effect was seen for dendrimer **I**.

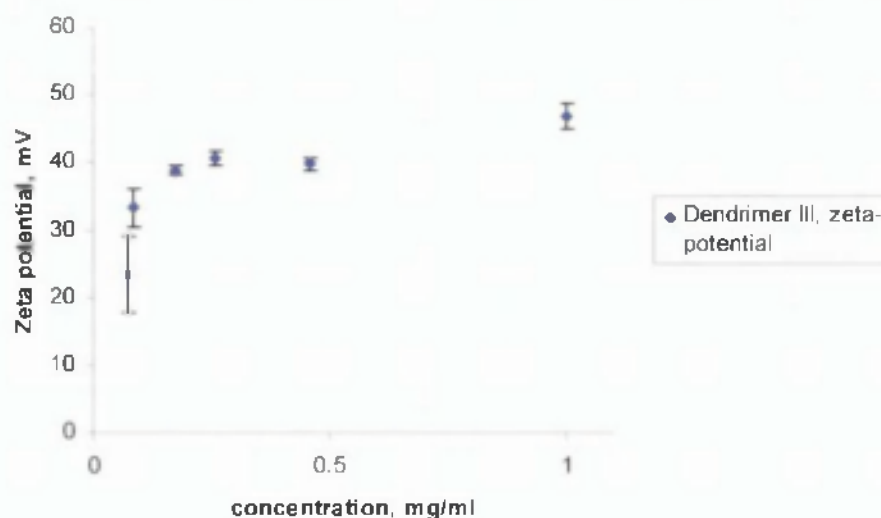


Figure 3.15. Effect of dendrimer III concentration on zeta-potential. The zeta-potential increases with the dendrimer concentration, reaching a plateau at the higher concentrations (n=3).

3.3.4 Calculation of packing

A calculation was made to help understand the decrease in particle diameter of the nanoparticles as the dendrimer concentration increased (dendrimer II and III). A few assumptions were made to simplify the calculations and for illustration purposes:

- the dendrimers were spherical in shape
- they were cubic/hexagonal closed packed
- interaction between the dendrimers was through the lipidic chains
- the overlap between the surface lipidic chains of the dendrimers was half the length of the lipidic chain, to show the possible effect of the interaction on the size of the aggregates

The model showed the possible effect of packing on the particle diameter.

Assuming that the dendrimers are spherical, the densest packing possible is cubic and hexagonal close packed structures which have the same packing densities. Calculations using either model give the same values (Conway and Sloane 1993). The theoretical calculation here are based on cubic close packing. The packing density of the dendrimers nanoparticles was calculated. Firstly assuming they were hard spheres in order to calculate the volume they occupy with no interaction and secondly, assuming that the interaction is through the lipidic chains and is approximately half the length of the lipidic chains. In Table 3.6 the diameter of the dendrimers (I, II and III) has been listed together with the length of the lipidic chains and the diameter of the 'core' of the dendrimers. The data was estimated from the molecular models as explained previously in section 2.3.1. The dendrimer 'core' represents the dendrimer diameter excluding the surface lipidic chains.

Table 3.6. The diameter of dendrimers I, II and III, their respective carbon chain lengths and "core" diameters.

Dendrimer	Diameter, nm	Carbon chain length, nm	Dendrimer 'core' diameter, nm
I	4.7	0.6	3.5
II	6.0	1.2	3.6
III	6.6	1.6	3.4

If firstly, the dendrimers are assumed to be hard spheres and cubic closed packed, the total packing density can be calculated. Considering the cubic close packing of 14 dendrimer molecules these can close pack as illustrated in Figure 3.16. when arranged as shown in Figure 3.16, the spheres form a cube, consisting of $8 \times \frac{1}{8}$ spheres (a) and $6 \times \frac{1}{2}$ spheres (b).

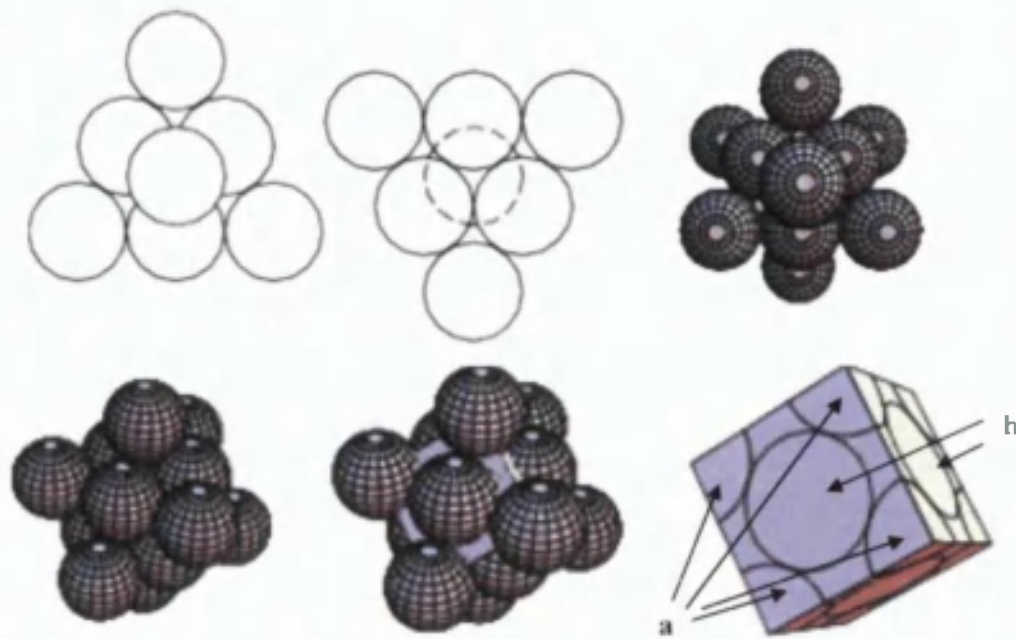


Figure 3.16. Illustration showing cubic close packing using 14 spheres. When arranged as shown, the spheres form a cube containing $8 \times \frac{1}{8}$ spheres (a) and $6 \times \frac{1}{2}$ spheres (b) (Steinhaus 1999).

From one of the square planes of the cube, the total volume of the cube can be calculated, by determining the length of each of the two sides in the square.

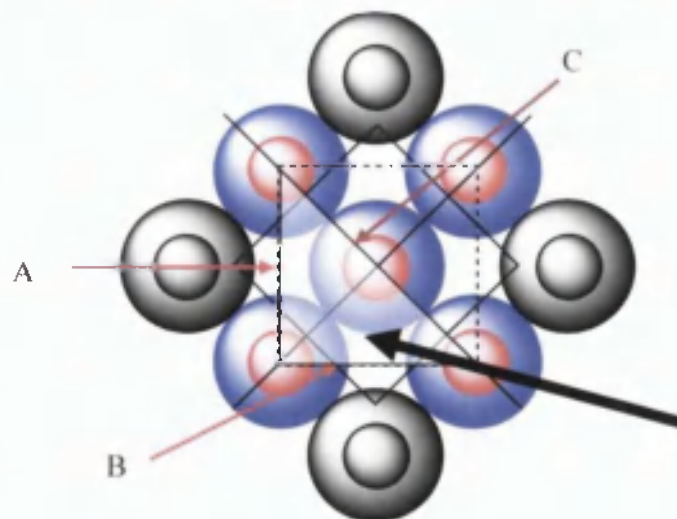


Figure 3.17. This diagram demonstrates one square of the cube (----) from which a triangle is made which is illustrated by the black arrow. Using Pythagoras the length of the sides of the triangle can be calculated: $A = B = 2\sqrt{2}r$ and $C = 4r$.

Making a triangle in the square (Figure 3.17), the length of the two sides can be calculated using Pythagoras:

$$\begin{aligned}
 & A^2 + B^2 = C^2, A = B, \text{ and } C = 4r \Rightarrow \\
 (1) \quad & A^2 + A^2 = 4r^2 \Leftrightarrow 2A^2 = 16r^2 \Leftrightarrow A = 2\sqrt{8}r \Leftrightarrow \\
 & A = 2\sqrt{2}r = B
 \end{aligned}$$

This means that the length of each side in the square is determined by equation 1.

From eq. 1 the total volume of the cube (V_{cube}) was calculated:

$$\begin{aligned}
 (2) \quad & V_{\text{cube}} = (2\sqrt{2}r)^3 \Leftrightarrow 8 \times 2\sqrt{2}r^3 \Leftrightarrow \\
 & V_{\text{cube}} = 16\sqrt{2}r^3
 \end{aligned}$$

Now consider only the spheres in the cube. As mentioned before the cube will have 8 x 1/8 spheres and 6 x 1/2 spheres. The total volume of the spheres is therefore:

$$\begin{aligned}
 (3) \quad & V_{\text{spheres}} = (8 \times \frac{1}{8} + 6 \times \frac{1}{2}) \times \frac{4\pi r^3}{3} \Leftrightarrow 4 \frac{4\pi}{3} r^3 \Rightarrow \\
 & V_{\text{spheres}} = \frac{16}{3} \pi r^3
 \end{aligned}$$

The packing density was therefore calculated by:

$$(4) \quad \frac{V_{\text{spheres}}}{V_{\text{cube}}} = \frac{\frac{16}{3} \pi r^3}{16\sqrt{2}r^3} = 0.74$$

This means that the packing density of the spheres is 74% in the cube (Steinhaus 1999, Wells 1986). The packing density of the dendrimers aggregates was then calculated. The length of lipidic chain (DIII, C₁₂) is ~1.6nm in vacuum. Assuming that the dendrimer is completely spherical and that the lipidic chains extend in all directions, the dendrimer diameter was estimated to be 6.6nm using molecular modeling. Assuming that the interaction between the dendrimers is through the lipidic chains there will be a total increase in volume of spheres in the square (Figure 3.18).

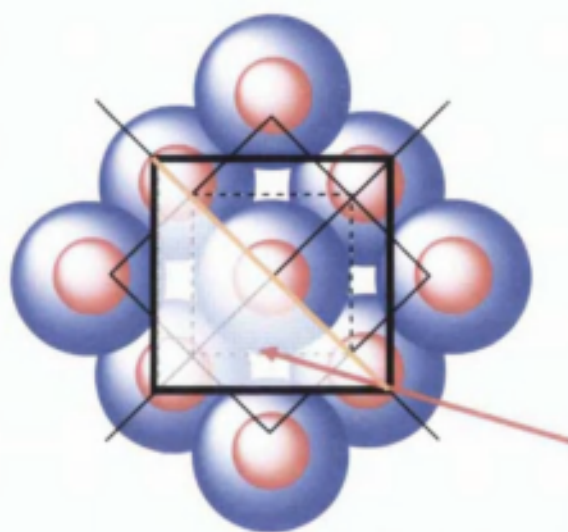


Figure 3.18. This diagram shows an increase in density of spheres in the square (—). The overlap of dendrimers on the length of the diagonal (—) of the white triangle (—) was $(1.6 \text{ nm} + 1.6 \text{ nm}) / (2 \times 13.2 \text{ nm}) = 12 \%$.

Considering the diagonal (—) of the white triangle (—) in Figure 3.13, the total overlap (diagonal) in length is therefore $(1.6\text{nm} + 1.6\text{nm}) / (2 \times 13.2 \text{ nm}) = 12 \%$. Converted into volume this is an increase of approximately one quarter of a sphere for each corner ($8 \times 1/4$) in the cube (Figure 3.19, represented by the thick black line). And a further $12 \times 1/107$ spheres are condensed into the cube (Figure 3.19, represented by the brown color).

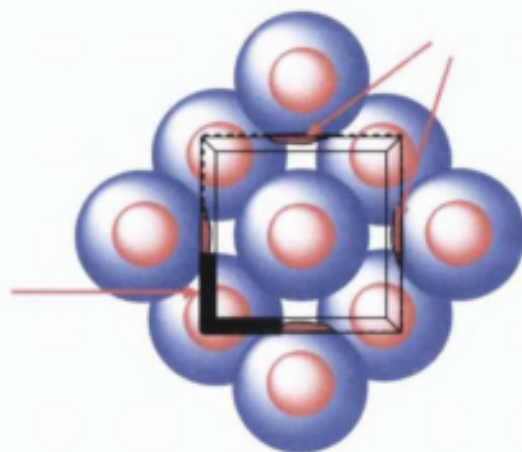


Figure 3.19. This diagram shows the increase in volume of the spheres in the square. The black colour showing an increase of approximately one quarter of a sphere for each corner ($8 \times 1/4$) in the cube. A further $12 \times 1/107$ is condensed in to the square (Brown colour).

The total number of spheres in the cube therefore increases to:

$$(8 \times \frac{1}{8} + 6 \times \frac{1}{2}) + 8 \times \frac{1}{4} + 12 \times \frac{1}{107}$$

The total volume of aggregated spheres in the cube was then calculated.

$$(5) \quad V_{agg\ spheres} = ((8 \times \frac{1}{8} + 6 \times \frac{1}{2}) + 8 \times \frac{1}{4} + 12 \times \frac{1}{107}) \frac{4\pi r^3}{3} \Leftrightarrow 6.12 \frac{4\pi}{3} r^3 = 8.14 \pi r^3$$

$$(6) \quad \frac{V_{agg\ spheres}}{V_{cube}} = \frac{8.14 \pi r^3}{16\sqrt{2}r^3} = 1.13$$

The total theoretical volume of the spheres exceeds that of the cube. That is why the density is more than 1. This is of course only possible if the dendrimers overlap each other.

The total increase in density is therefore

$$V_{\text{density}} = 1.13 - 0.74 = 39\%$$

Using the same method the total theoretical increase in density has been calculated for dendrimer I and II and is listed in table 3.7.

Table 3.7. The total changes in the diameter observed for dendrimer-derived nanoparticles: experimental and theoretical

	% change (theoretical)	% change (experimental)
Dendrimer I	15% ↓	462% ↑
Dendrimer II	31% ↓	56% ↓
Dendrimer III	39% ↓	61% ↓

This theoretical calculation shows that a decrease in particle size is possible if there is lipidic interaction between the dendrimers. The theoretical values calculated are lower than the actually achieved values (dendrimer II and III). This difference is most probably because the dendrimers are not completely spherical, which was one of the assumptions in these calculations, but also the fact that the interaction was assumed to be half the length of the lipidic chain. The results showed, that although, theoretically, a decrease was possible for dendrimer I it did not fit with the experimental data, suggesting that flexibility of lipidic chains changes with the carbon chain length and hence behave differently when interacting through the lipidic chains.

3.3.5 Effect of non-ionic surfactant on particle diameter

The assembly of dendrimers seemed to be through the lipidic chains on their surfaces. As discussed previously in this chapter, the dendrimer-derived nanoparticles had a particle distribution. The larger particles ($> 100\text{nm}$) contributing $\sim 1.5\%$ in the 5th generation dendrimer-derived nanoparticles and 10% in the 6th generation dendrimer-derived nanoparticles. The addition of surfactant to the water phase would prevent any coalescence of droplets during the formulation of particles. The effect of surfactant on dendrimer-derived nanoparticles size was therefore assessed. The particles were formulated as described in section 3.2, method 3. The concentration of dendrimer was kept constant at 0.1% w/v and the effect of a nonionic surfactant (polyoxyethylene cetyl ether E10, $\text{CH}_3(\text{CH}_2)_{15}(\text{OCH}_2\text{CH}_2)_{10}\text{OH}$) was assessed (Figure 3.20). Increasing the surfactant concentration from 0.5% w/w to 5% w/w resulted in an increase in the mean number average diameter of the formed nanoparticles (of dendrimer I, 24nm to 44nm , of dendrimer II and 30nm to 66nm , dendrimer III, 24nm to 56nm) (Figure 3.21) the larger particles ($>100\text{ nm}$) contributing to $\sim 1.5\%$ of the total amount of dendrimer-derived nanoparticles. A value similar to dendrimer-derived nanoparticles formulated without any surfactant. This increase in particle diameter seems to be the direct effect of the interaction between the lipidic chain of the dendrimer and the lipidic chain group of the surfactant. As mentioned previously the external amino groups of the dendrimers are modified with different lengths of lipidic chains and provide the basis for hydrophobic interactions between the two. Dendrimer II, with C_{10} lipidic chains, forms larger dendrimer-derived nanoparticles with surfactant compared to particles formed with dendrimer I and III. The data correlates with the data shown in section 3.3.2, where the formulation of dendrimer-derived nanoparticles without surfactant

showed the dendrimers with C₁₀ lipidic chains attached to the terminal amino groups to form larger dendrimer-derived nanoparticles by number values. The reason for this effect, most probably lies in the structural flexibility of the lipidic chains and the more extended nature of dendrimer II in an aqueous environment. Considering the core of the dendrimers to be ridged, the lipidic chains on the terminal amino groups account for flexibility, hydrophobicity and hence the dendrimer interaction. The hydrophobicity is dependent on the length of the lipidic chain, and the flexibility dependent on carbon chain length and their physical state in an aqueous environment. A short carbon chain length on the dendrimer surface seems ridged. As the carbon chain length increases the flexibility increases. At a carbon chain length above 10, the shape of the lipidic chain seems to change, resulting in smaller dendrimer-derived nanoparticles of dendrimer III, with C₁₂ lipidic chains, compared to particles formed from dendrimer II, with C₁₀ lipidic chains. The critical limit for the structural changes seems to be a carbon chain length larger than 10.

Comparing the results with the dendrimer-derived nanoparticles formulated without any surfactants as described in section 3.3.3 (where the dendrimer concentration was increased), it is evident that packing and hence particle diameter are linked to the flexibility and hydrophobicity of the dendrimers. Combined with the molecular models of the dendrimers (Chapter 2, Figure 2.7) and the spacefilling model of the surfactant it is likely that the surfactant interferes with the flexible surface lipidic chains of the dendrimer-derived nanoparticles. No increase in stability of the dendrimer-derived nanoparticles were found using the surfactant as the polydispersity of the system still remained the same.

Given the fact that dendrimers on its own has surface active properties, addition of surfactant was not useful. For the further experiments no surfactants were used, as they did not increase particle stability and most importantly they altered the surface properties of the dendrimer-derived nanoparticles. Hydrophobic dendrimer-derived nanoparticles, with their flexible lipidic chains, were formulated to target Peyer's patches of the GALT. Changes in the delivery system would complicate the assessment of the carrier

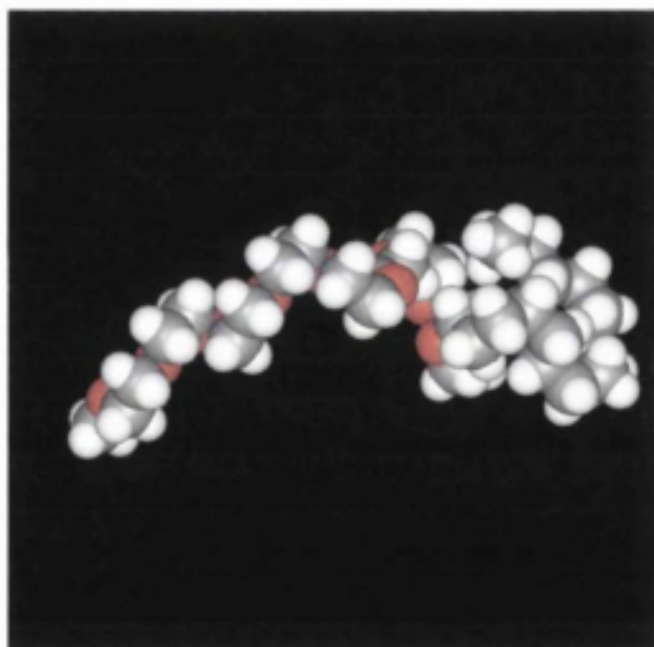


Figure 3.20. Illustration of a space-filling model of the of polyoxyethylene cetyl ether E10, $\text{CH}_3(\text{CH}_2)_{15}(\text{OCH}_2\text{CH}_2)_{10}\text{OH}$.

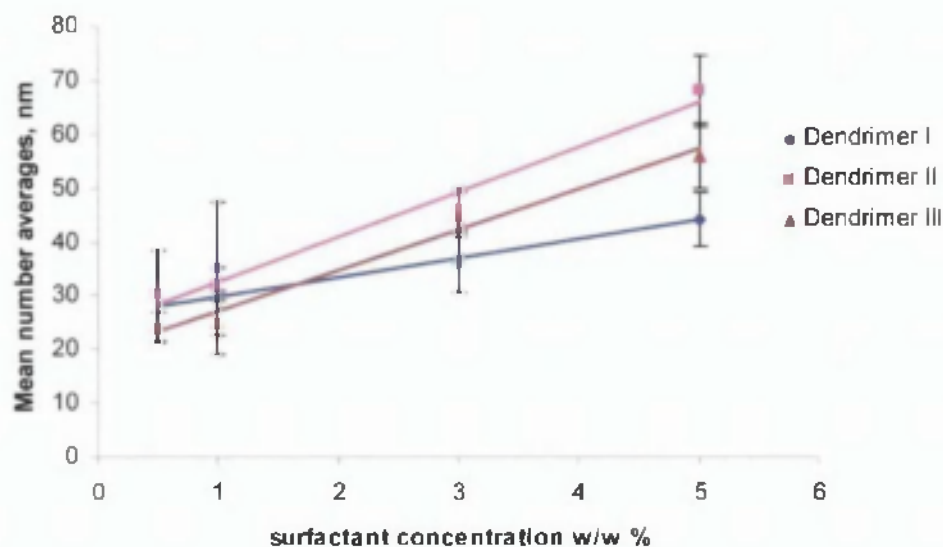


Figure 3.21. The effect of surfactant concentration ($\text{CH}_3(\text{CH}_2)_{15}(\text{OCH}_2\text{CH}_2)_{10}\text{OH}$) on the mean number diameter of dendrimer-derived nanoparticles. Increasing the surfactant concentration increases the mean number diameter ($n=3$).

3.3.6 Investigating packing using pyrene as an hydrophobic probe

Pyrene (excitation, 340nm) is a hydrophobic fluorescent probe. The intensity (peak height) ratios of the first band (374nm) to the third band (385nm) (I_1/I_3) in the emission spectra (350-600nm) were determined as a function of dendrimer (**II** and **III**) concentration (Figure 3.22). It is well known that the ratio between the first and the third vibronic peak (I_1/I_3) is proportional to the polarity of the medium in which it is dissolved. Results show a decrease in I_1/I_3 , indicating an increase in the non-polar nature of the dendrimer aggregates as concentration increased. A plateau is reached at the higher concentration suggesting that a compact form of the aggregates was attained.

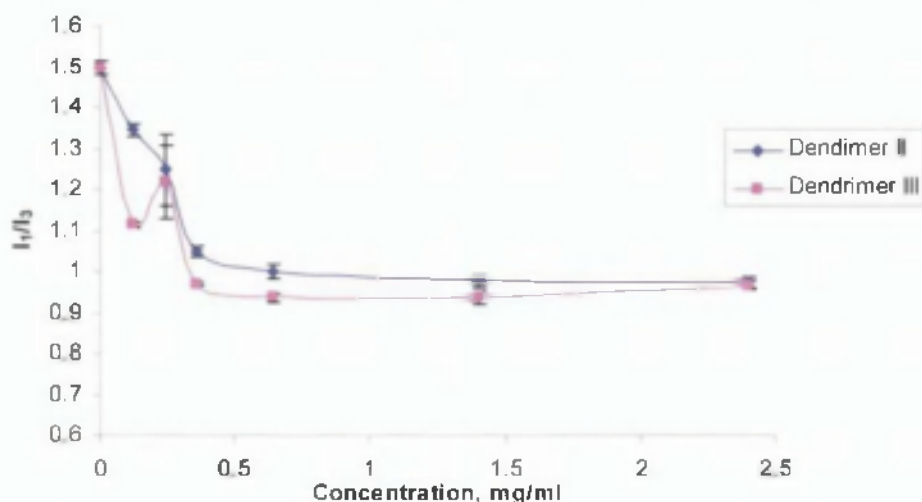


Figure 3.22. The effect of dendrimer (II and III) concentration on I_1/I_3 ratios investigated using pyrene (excitation, 340nm) as a hydrophobic fluorescent probe. The intensity (peak height) ratios of the first band (374nm) to the third band (385nm) (I_1/I_3) in the emission spectra (350-600nm) are plotted here as a function of dendrimer concentration ($n = 3$).

For surfactants a classic inverted s-shape (Figure 3.23) is normally obtained. The sharp decrease indicating micelle formation from which critical micelle concentration can be determined (Esumi *et al.* 2002; Bakshi 2004).

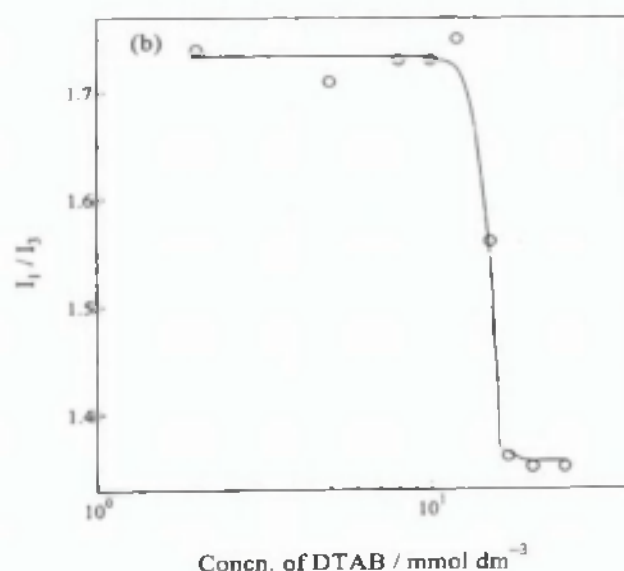


Figure 3.23. I_1/I_3 of pyrene versus logarithm of surfactant concentration for dodecyltrimethyl ammonium bromide (DTAB) (Esumi *et al.* 2002).

Comparing the s-shape curve in Figure 3.23 to the curve obtained in the presented data, Figure 3.22, it is apparent that the initial plateau is missing. That is because the plateau is only evident in systems measuring polarity significantly below the CMC. In the experiment conducted in this chapter, it is the polarity of the system after particle formation that is measured and that is why the initial lag phase is not apparent.

As the dendrimer-derived nanoparticles are assembling, a decrease in polarity (I_1/I_3) is observed. A further increase in concentration results in a second plateau. The resulting plateau is a conformation of the formation of “complete” dendrimer-derived nanoparticles (Figure 3.24). The results suggest that a concentration of 0.36 mg/ml (dendrimer II) and 0.22 mg/ml (dendrimer III) is the concentration at which particles are completely formed. Dendrimer II, with its C_{10} lipidic chains, resulted in a higher I_1/I_3 ratio than for dendrimer III with C_{12} lipidic chains. The results confirmed that a more hydrophobic dendrimer resulted in a more hydrophobic environment.

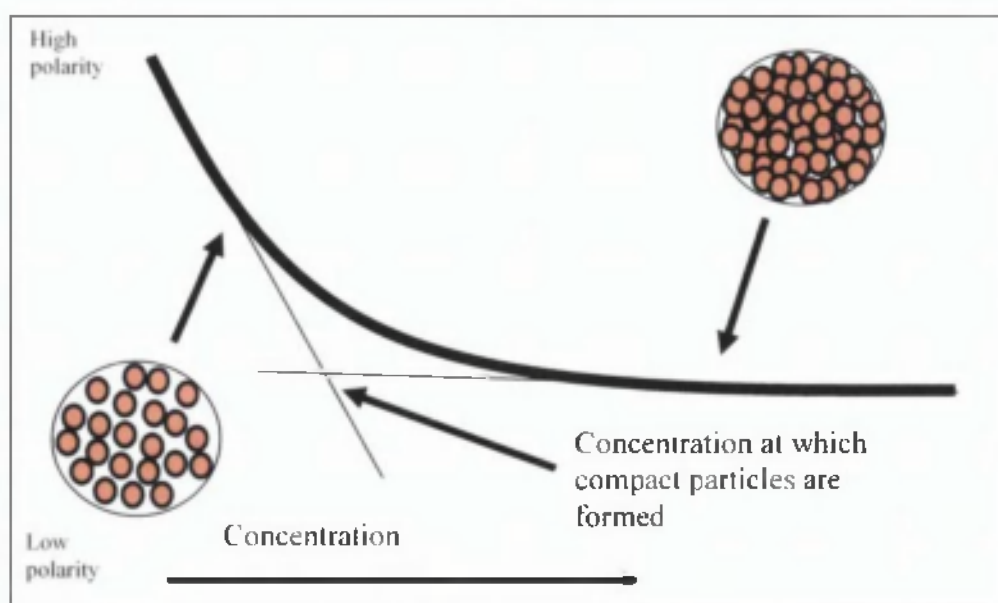


Figure 3.24. Schematic representation of the formation of dendrimer-derived nanoparticles and the effect this presence on polarity.

The results suggested that dendrimers with longer lipidic chains, formed compact aggregates at a lower concentration. The assembly of dendrimer-derived nanoparticles was not only dependent on surface properties of the dendrimer, but also on dendrimer concentration. The dendrimer concentration determines the compactness of the nanoparticles. The mode of assembly is important when considering stability of the nanoparticles but also for potential drug entrapment. Altering the compactness of the dendrimer-derived nanoparticles is very likely to affect drug entrapment; “loose” nanoparticles might allow higher drug entrapment because of the larger void spaces available. For hydrophobic drugs “loose” or tightly packed particles would be useful, as the drugs would interact with the surface lipidic chains of the dendrimers and contribute to the aggregation and hence the formation of dendrimer-derived nanoparticles. The circumstances depend on the application.

3.4 Conclusion

It was possible to formulate a stable aqueous suspension of dendrimer-aggregates for oral delivery. The formation of nanoparticles is mainly governed by the hydrophobic surface nature of the dendrimers with the charge of the particles contributing to the stability of the particles in suspension. The particle diameter was predominantly governed by the dendrimer generation. Surface modification of the dendrimers with C_4 to C_{12} lipidic chains resulted in the following ranking in the diameter of the derived nanoparticles $C_{10} > C_{12} \approx C_4$. Nanoparticles from dendrimer (II) with C_{10} surface lipidic chains formed larger particles. Monolayer studies of the dendrimers at the air/water interface showed they formed a more expanded monolayer than the other dendrimers in their generation and hence their ‘looser’ packing into nanoparticles resulted in a larger diameter.

The increase in the dendrimer generation showed that the 6th generation dendrimers formed larger dendrimer-derived nanoparticles than 5th generation dendrimers, an effect mainly due to the larger diameter of the individual dendrimers. However, studies at molecular level showed that the 6th generation dendrimers formed a more expanded monolayer at the air/water interface and hence formed larger dendrimer-derived nanoparticles when assembled together. The experiments underline the importance of studying dendrimers at molecular level in order to understand the parameters influencing their aggregation into nanoparticles.

By changing the dendrimer concentration it was possible to control the particle diameter. Increasing the dendrimer concentration for dendrimers with surface lipidic chains C₁₀ and C₁₂ decreased the size of dendrimer-derived nanoparticles while a shorter carbon chain length (C₄) resulted in an increase in particle size. Calculations of packing suggested that the carbon chains provided space, in which the dendrimers could interact. Combined with the flexibility of the lipidic chains these features resulted in a concentration dependent packing. The packing was governed by surface flexibility and hydrophobicity of the dendrimers, with short carbon chains on the dendrimer surfaces, providing a more ridged molecule and hence an increase in particle diameter was observed as the dendrimer concentration increased. Measuring the length of the surface carbon chain resulted in more compact aggregates at a lower dendrimer concentration due to increased hydrophobic interaction.

The size and stability of dendrimer aggregates was dependent on the generation of dendrimers, the concentration and the packing characteristics of the dendrimers, mainly the interaction between the hydrophobic surface groups. However, beside the formation of mainly smaller aggregates, larger aggregates of dendritic matter were

also observed. The main issue seems to be the control of additional larger aggregates. The potential of self-assembly of dendritic matter in drug, vaccine and gene delivery is still to be assessed and whether or not the relatively few larger aggregates obscures results, needs to be assessed for each individual case.

Chapter four

***In vitro* studies – stability and cell toxicity of dendrimer aggregates**

4.1 Introduction

In this chapter dendrimer-derived nanoparticles, derived from dendrimers I to VI, were incubated with Caco-2 cells and red blood cell in order to assess their toxicity *in vitro*. The effect of dendrimer generation and various surface lipidic chain lengths was investigated in order to investigate the biocompatibility of the synthesised dendrimers and various cell lines. These studies are a prerequisite prior to *in vivo* studies for animal safety reasons, but also in search for information about the interaction between dendrimer-derived nanoparticles and the cell membrane. Furthermore, the stability of dendrimer-derived nanoparticles was assessed in relevant biological media, to study the physical properties of dendrimer-derived nanoparticles prior to uptake.

The physical properties of dendrimers in this thesis have been characterized by mass spectrometry, NMR, Langmuir trough and molecular modeling and dendrimer derived nanoparticles by zeta potential, particle sizing, polarity measurements and transmission electron microscopy in order to understand the interaction of such particles with mammalian cells. Dendrimers have potential in tissue targeting applications, controlled drug release and because of their nanoscopic architecture and diverse structural features can be modified perhaps to reduce any cytotoxicity, enhance stability in biological media and optimise uptake across biological barriers.

4.1.1 Dendrimer toxicity

For *in vivo* applications it is essential that the carriers show no or very low, toxicity. Most drugs given in high enough doses show a toxic effect, and therefore it is important when conducting *in vitro* experiments to keep in mind the difference in scale, with the area of a 96 well plate ~ 400 times smaller than that of the epithelial lining in the intestine.

Cationic dendrimers are known generally to exhibit cytotoxicity. Polyamidoamine (PAMAM) dendrimers with terminal amino groups, have shown concentration and generation dependent haemolysis and cause changes in red cell morphology. Furthermore, cationic dendrimers displayed cytotoxicity with a concentration and generation related variation, but also showed that dendrimer type and cell type contributed to the variation in the cytotoxicity (Malik *et al.* 2000). Cytotoxicity of a range of polycations has been tested (Fischer *et al.* 2003) and ranked as follows regarding their cytotoxicity: poly(ethylenimine) = poly(L-lysine) > poly(diallyl-dimethyl-ammonium chloride) > diethylaminoethyl-dextran > poly (vinyl

pyridinium) bromide > starburst dendrimers > cationised albumin > native albumin. Key parameters for the interaction with cell membranes and consequently cell damage were time, concentration, molecular weight and the degree of cationic charge density with high cationic charge densities resulting in higher cytotoxicity of the polycations.

The effect of charge density on the cell membrane, was suggested to be an effect of flexibility and density of their three dimensional structure. It was argued, that the activity of amino groups on the cell membrane decreases when the space between reactive amine groups increased (Ryser 1967). The arrangement of cationic charges depends on the three-dimensional structure and flexibility of the macromolecules which affects the access of charges on the cell surface. For example, branched molecules were found to be more efficient in interacting with the cell surface charge than polymers with linear structure, as ridged molecules have more difficulties to attach to the membranes than flexible molecules (Singh *et al.* 1992). Therefore, high cationic charge densities and highly flexible polymers should cause higher cytotoxic effect than those with low cationic charge densities.

As most of these assays are conducted in cell culture media such as PBS or HBSS, it is important to consider the effect of salts in these media on the cationic charge of these polycations. The media in which they are suspended, might ameliorate the cytotoxic effects. This would be considered a positive effect, but it is important to note that the conclusions have to be carefully made, as a cationic dendrimer in cell culture media might not have an over all positive charge. Malik *et al* (2000) showed, that dendrimers with carboxylate terminal groups showed much lower haemolytic or cytotoxic effects towards a panel of cell lines. Furthermore, conjugation of PAMAM

dendrimer with PEG show a decrease in toxicity (Luo *et al.* 2002). In this chapter we have examined the haematotoxicity and *in vivo* cytotoxicity of lipidic dendrimers

4.1.2 Stability of dendrimers prior to uptake

Only a few studies have been carried out on the physical properties of particles in cell culture media. One such study by Pouton *et al.* (1998), showed that cationic poly(amino acid)/DNA complexes when diluted into opti-MEM media, were unstable to electrolyte challenge. The apparent particle size increased, and it was proposed that this particle growth represented aggregation due to changes in the electrical double layer, lowering the zeta potential, compressing the electrical diffuse layer and leading to reduced charge repulsion between the particles. We (Singh *et al.* 2003) also showed in a separate study, that increasing the ionic strength of the media significantly increased the apparent size of the DNA-dendron-gold particles. Importantly, in cell culture media the size of the DNA-dendron-gold nanoparticles increased markedly, as surface potential was reduced. The presence of serum components partially ameliorated these effects, possibly due to steric stabilization of the particles after adsorption of proteins.

Not only electrolytes affect the particles. Serum albumin has also been shown to bind to poly-L-lysine/DNA complexes forming a ternary poly-L-lysine/DNA/albumin complex (Dash *et al.* 1999). This indicates that biological media, which contains a wide range of salts, hormones, growth factors, vitamins, enzymes and serum proteins are complex media. There are indeed a multitude of factors which determine the physical properties of particles. Increased stability of carriers in biological media would provide enhanced protection of encapsulated drug but importantly prevent

interpretation of experiments being misleading because of the change in medium. In this chapter the stability of selected dendrimers has been assessed in simulated and purified gastric and intestinal fluid.

4.2 Materials and Methods

4.2.1 Materials

Dendrimers I to VI, described in chapter 2, were used in the studies. Colonic carcinoma cells, Caco-2 cells (passage 21), were obtained from European Collection of Cell Culture (ECACC), Wiltshire, UK. Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum, glutamine, non-essential amino acids, penicillin and HBBS were all obtained from Gibco BRL, Life Technologies, UK. Tissue culture flasks (TPP, Switzerland) were purchased from Nalgen Nunc, USA. Blood was taken through cardiac puncture from Female Sprague Dawley rats was supplied from Harlan, Ltd, UK and lithium-heparin tubes (4.5 ml, 75 x 13 mm) were purchased from Sarstedt, Germany. Filters (0.6 and 0.2 μm) were obtained from Millipore, Billerica, USA. SnakeSkin dialysis membranes (MWCO 3500), 22 mm x 35 feet dry diameter (34 mm dry flat width) 3.7 ml/cm were obtained from Pierce, USA. Optiphase 'Safe' from Wallac, Finland (formulated and produced by Fisher chemicals, UK). Triton X-100, optical grade dimethyl sulphoxide (DMSO), 3-[4,5-Methylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) purchased, 96 well flat bottom plate, hexamethyl disilazane (HMDS), osmium tetroxide (2%), glutaraldehyde, pancreatin, pepsin, potassium phosphate, sodium hydroxide, hydrochloric acid and phosphate buffered saline tablets (PBS, pH 7.4) from Sigma (UK). Ethanol and DCM were obtained from BDH, Leicestershire, UK.

4.2.2 Methods

4.2.2.1 Preparation of dendrimer-derived nanoparticles

The dendrimer derived nanoparticles were prepared as described in chapter 3, section 3.2.1, Method 3. Dendrimer-derived nanoparticles were formulated using a dendrimer concentration of 1.7 mg/ml at which an average particle diameter of ~25 nm was obtained (Chapter 3). A size which was also chosen for *in vivo* studies (chapter 5). Aliquots were taken from the dendrimer suspension and incubated with Caco-2 cells, RBC, purified intestinal and gastric fluid and simulated intestinal and gastric fluid as described in the following section.

4.2.2.2 In vitro cytotoxicity assay

The *in vitro* cytotoxicity of dendrimer-derived nanoparticles was studied using the MTT (mitochondrial dehydrogenase) assay. The MTT assay depends upon the reduction of the yellow tetrazolium salt, MTT by mitochondrial dehydrogenase of viable cells to a blue formazan product. The assay measures the cell respiration and the amount of formazan produced is proportional to the number of living cells present in the assay (Mosmann 1983).

Caco-2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 2mM glutamine, 10% (v/v) foetal bovine serum, 10mM non-essential amino acids and 50 IU/ml penicillin, at 37 °C and an atmosphere of 5% CO₂. The growth medium was changed every 2-3 days and cells counted by using trypan blue analysis. Aliquots of cell suspension were added to equal amount of trypan blue solution (2 % v/v) and the average number of viable cells was estimated using an

haemocytometer. The total number of viable cells/ml was calculated as: cell count per square x 2 (dilution factor) x 10^4 . After passage 32, Caco-2 cells were seeded at a density of 10^5 cells per ml in a 96 well plate and grown for 24h before addition of fresh media containing dendrimers. The cells were then incubated with aliquots of dendrimer I to VI solutions, concentration 1.71mg/ml, for 1.5h (n=5). After 1.5h MTT (5 mg/ml, 20 μ l) was added and left for 3h after which the media was removed and DMSO, 100 μ l, was added to dissolve the MTT crystals. After half an hour incubation, the optical density at 570nm was measured using a Labsystems Multiskan Multisoft plate reader. Results were expressed as the percentage viable cells, cells grown in the presence of dendrimers / cells grown in the absence of dendrimers.

4.2.2.3 Haematotoxicity assay

The haematotoxicity of dendrimer-derived nanoparticles were studied using a standard red blood cell assay (Malik *et al.* 2000). The study was conducted in order to investigate the toxic effect of dendrimer-derived nanoparticles on red blood cells.

Female Sprague Dawley rats (150g) were sacrificed using CO₂ asphyxiation and blood obtained by cardiac puncture. The blood was collected in heparin/lithium tubes to minimize coagulation. Erythrocytes were isolated by centrifugation at 1000 x g for 10 min at 4 °C and the supernatant was discarded. The RBC pellet was resuspended with prechilled (4°C) PBS at pH 7.4. The RBCs were again subjected to centrifugation and the supernatant discarded again. The pellets were weighed and resuspended in PBS to a 2 % (w/v) RBC suspension.

Aliquots of the 2% w/v RBC suspension was added to aliquots of dendrimer solution and PBS at pH 7.4 added to a total volume of 200 μ l. This was performed in 96 well flat bottom plates. The plates were incubated under standard conditions for 1.5h. Aliquots of the RBC suspension was incubated with PBS to generate a negative control. Incubation with 1% v/v solution of Triton X-100 was used to release 100% haemoglobin.

After incubation of the plates the content of the wells were transferred to Eppendorf tubes and subjected to centrifugation at 1500 x g for 15min. at room temperature. 100 μ l of the supernatant was then carefully removed without disturbing the pellets at the bottom of the Eppendorf tube and transferred into clean plates. The absorbance was measured at 550nm to assess the degree of haemolysis relative to the Triton X-100 control. The results were expressed as haemoglobin release (% +/- SD) as a function of the dendrimer concentration at pH 7.4 (n=5).

4.2.2.4 Stability of dendrimer-derived nanoparticles in simulated intestinal and gastric fluid

The dendrimer-derived nanoparticles, derived from dendrimer III were incubated with simulated gastric and intestinal fluid, inside a dialysis bag, to study the stability of dendrimer-derived nanoparticles prior to uptake (Figure 4.1).

Simulated intestinal and gastric fluid were formulated according to the United States Pharmacopoeia (USP, 2001): *Simulated intestinal fluid*: 68 g of monobasic potassium phosphate was dissolved in 250ml of water, and mixed. 190 ml of 0.2 N sodium hydroxide and 400 ml water was added. After addition of pancreatin (10g) the resulting solution was adjusted to pH 7.5 ± 0.1 with 0.2 N sodium hydroxide. The

solution was then diluted with water to a total volume of 1000ml. *Simulated gastric fluid*: 2.0 g sodium chloride and 3.2 g of pepsin was dissolved in 7.0 ml of hydrochloric acid and sufficient water added to make up 1000ml. The pH of the solution was ~1.2.

One mixture was made up with enzymes for the internal solution and one without for external solution (Figure 4.1). Inside the dialysis bag (MWCO 3500), 5 ml of 'internal' solution was incubated with 2 ml of dendrimer derived nanoparticles (1.71 mg/ml), derived from dendrimer III. The contents of the dialysis bag were stirred at gentle speed and samples from the external media was taken and the radioactivity of each sample was measured as described in section 4.2.2.7.

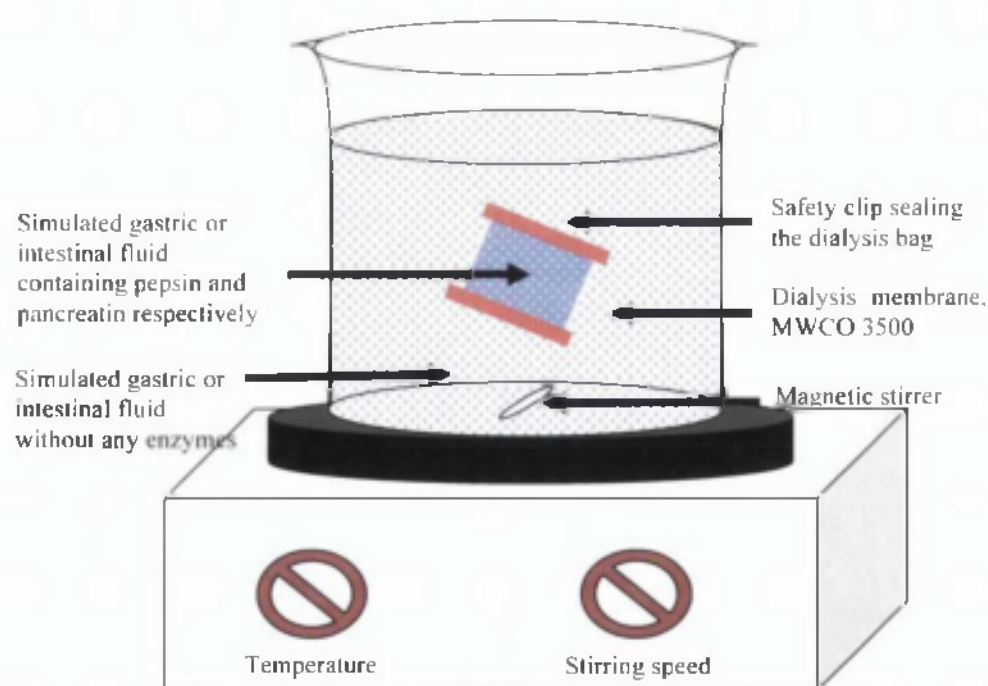


Figure 4.1. The experimental setup for the dialysis assay. One mixture contained enzymes for the internal solution and one without for the external solution. Inside the dialysis bag, simulated intestinal or gastric fluid with enzymes was incubated with dendrimer derived nanoparticles. The dialysis bag was stirred at gentle speed and samples from the external medium were taken and the radio activity of each sample was measured.

4.2.2.5 Stability of dendrimer-derived nanoparticles in purified intestinal and gastric fluid

The study of physical properties of particles prior to uptake are important, if accurate predictions and conclusions are to be made. Dendrimer-derived nanoparticles were incubated with purified intestinal and stomach fluid to study the effect of biological media on the particle diameter.

Female Sprague Dawley rats (~150g) were sacrificed using CO₂ asphyxiation. The rats were dissected and the small intestine separated from the stomach and large intestine and flushed with double distilled deionised water (15 ml). The content was centrifuged and filtered sequentially through 0.6 and 0.2 µm filters. The stomach was cut open and the content washed in pure water (15ml), centrifuged and filtered through 0.6 and 0.2 µm filters.

The experiments were carried out by incubating 150 µl of a 1.71 mg/ml solution of dendrimer-derived nanoparticles, derived from dendrimers II and III, with purified intestinal (2 ml) and stomach fluid (2ml). The apparent particle size of the dendrimer-derived nanoparticles was then measured over a time period of 3 h. The pH of purified intestinal and stomach fluid was 6.5 and 4.8 respectively.

4.2.2.6 Particle size measurements

Dendrimer-derived nanoparticles, 1.71 mg/ml, derived from dendrimer I to VI, were formulated as described in section 3.2.1 (Method 3) and the apparent particle diameter was measured. For details see section 3.2.2.1.

4.2.2.7 Radioactivity measurements

Dendrimer III was radiolabelled with carbon (^{14}C) as described in section 2.4.2. For each individual sample, 100 μl was placed in a scintillation vial and made up to 5ml with Wallac OptiPhase HiSafe scintillation cocktail and placed in a Beckman LS 6500 multi-purpose scintillation counter. Counting was continued for 5 min, each sample measured three times, and the disintegrations per min (DPM) recorded.

4.2.2.8 Scanning electron microscopy

Scanning electron micrographs were taken using a Philips XL-30, Philips Co. (Eindhoven, Netherlands) microscope after drying on a Samdri 780 critical point dryer (Maryland, USA) and gold coating using a Emscope gold sputtering Coater, Ashford, UK.

4.2.2.8.1 Red blood cells

Scanning electron micrographs of RBC were obtained after incubation of RBC with aliquots of dendrimer-derived nanoparticles (1.71 mg/ml) (section 4.2.2.3). The RBC were prepared for imaging as described by Malik *et al.* (2000). The content of each well was transferred to sterile Eppendorf tubes and subjected to centrifugation at 1500 x g for 15 min at room temperature. The supernatant, plasma, was removed from the wells without disturbing the pellets, RBC, and discarded. A solution of electron microscopy grade fixative, glutaraldehyde, diluted in PBS (100 μl of a 2.5 % v/v) was then added to the wells and the RBC were resuspended. The suspension was transferred to an Eppendorf tube and fixated at room temperature for 18h.

The RBC were then subjected to centrifugation at 1000 x g for 2 min. at room temperature. The supernatant was again discarded and 100 μl of a 1 % (w/v) osmium

tetroxide (secondary fixative) diluted in sterile distilled water was added and the pellets were resuspended. The cells with the secondary fixative were left for another hour at room temperature.

The cells were again subjected to centrifugation and the supernatant was discarded. The cells were then dehydrated and proteins denatured with ethanol made up in sterile distilled water (100 μ l of 50 % v/v) for 5-10 min. at room temperature. The supernatant was again discarded after centrifugation. This step was repeated with 60 % (v/v) ethanol, 70 % (v/v) ethanol, 80 % (v/v) ethanol, 90 % (v/v) ethanol and 100 % (v/v) ethanol.

For scanning electron micrographs the RBC were chemically dried with hexamethyl disilazane (HMDS) (100 μ l) and the cells were subjected to gentle aspiration and left for 10 min at room temperature.

The aspirate was pipetted onto a glass coverslip and the HMDS was allowed to evaporate. The samples were mounted upon SEM platform using carbon cement and coated in gold and scanning electron micrographs were taken using a Philips XL-30 microscope.

4.2.2.8.2 Dendrimer-derived nanoparticles in purified intestinal and stomach fluid

The samples were dried (Samdri 780 critical point dryer) and gold coated using Emscope gold sputtering Coater. Scanning electron micrographs were taken using a Philips XL-30 microscope.

4.3 Results and discussion

4.3.1 Cytotoxicity of dendrimer derived-nanoparticles

Assessment of cell toxicity *in vitro* is a prerequisite prior to more extensive *in vivo* experimentation. Following oral administration, the main area of interaction is the intestinal epithelium. Therefore the toxicity of dendrimer-derived nanoparticles was assessed using a colonic carcinoma cell line (Caco-2). As nanoparticles ultimately can reach the systemic circulation (Eldridge *et al.* 1990, Rolland 1993), the toxicity and biocompatibility of dendrimer-derived nanoparticles was also assessed in red blood cells.

Because of the large interest in dendrimers for pharmaceutical applications, the toxicity of many dendrimers has been studied (Joester *et al.* 2003, Jevprasesphant *et al.* 2003, Malik *et al.* 2000, Roberts *et al.* 1996). There has however been conflicting evidence regarding their biological safety which in part has been contributed by the lack of consistency of experiments. The variation in cell lines and hence alteration in experimental conditions does make it very difficult to compare results. However, cationic PAMAM dendrimers have been shown to cause cytotoxicity in a panel of cell line, in a size, shape and concentration dependent manner (Jevprasesphant *et al.* 2003, Malik *et al.* 2000). Modifying cationic surface groups with lipidic chains, polyethylene glycol (PEG) seemed to shield the positive charge and decrease cytotoxicity (Jevprasesphant *et al.* 2003).

The cytotoxicity of dendrimer-derived nanoparticles, derived from lipidic dendrimers I to VI, synthesised and characterized in chapter 2 and 3, was assessed in concentrations from 23 µg/ml to 798 µg/ml (Figure 4.2 and 4.3). The 5th and 6th

generation dendrimers having surface aminogroups modified with lipidic chain (C_4 , C_{10} and C_{12}) showed a slight increase in cytotoxicity as the lipidic chain increased from C_4 to C_{12} . The IC_{50} (concentration that inhibits 50%) value for nanoparticles from dendrimers I, II, was more than 0.8 mg/ml and ~ 0.7 mg/ml for dendrimer III. For the 6th generation dendrimers, the IC_{50} values were more than 0.8 mg/ml for dendrimer IV and V, again however, the dendrimer with C_{12} surface lipidic chains, dendrimer VI, showed a slight increase in toxicity with IC_{50} values ~ 0.6 mg/ml

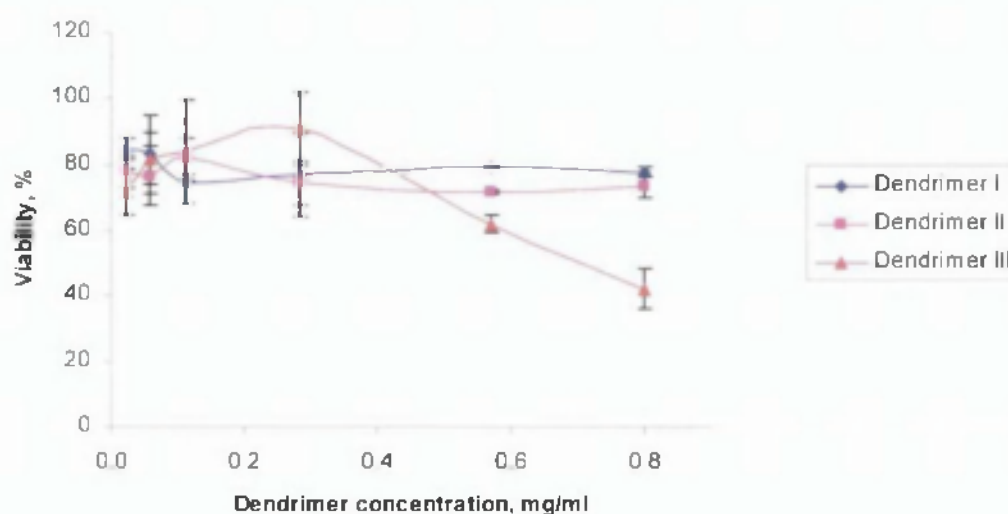


Figure 4.2. The effect of dendrimer-derived nanoparticles (derived from 5th generation dendrimers) on cell survival (mean $\pm$ SD, $n = 5$) as a function of concentration on Caco-2 cells. The graph shows the effect of an increase in surface lipidic chain on the cytotoxicity. At concentrations >0.54 mg/ml an increase in the length of the surface lipidic chain of the dendrimer (from C_4 to C_{12}), resulted in an increase in cytotoxicity of the dendrimer-derived nanoparticles.

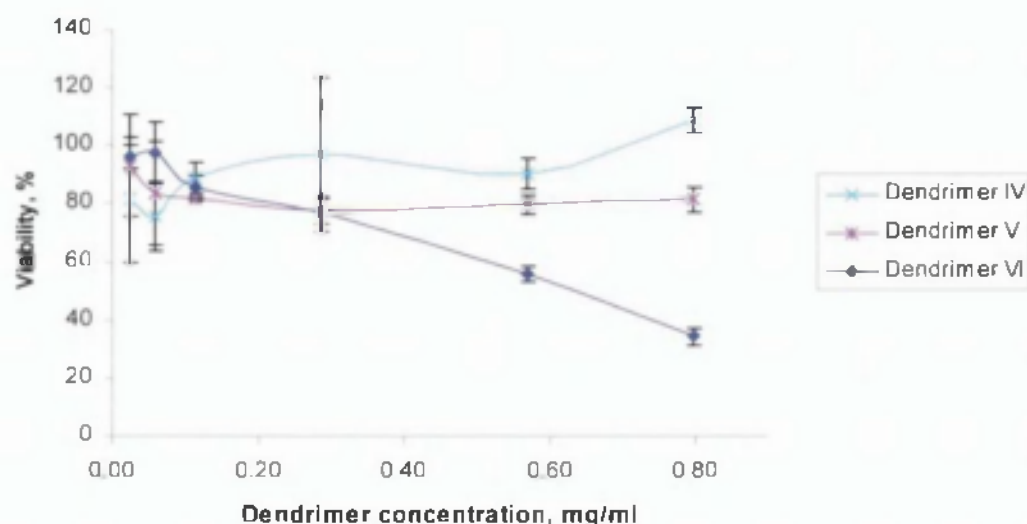


Figure 4.2. The effect of dendrimer-derived nanoparticles (derived from 6th generation dendrimers) on cell survival (mean $\pm$ SD, $n = 5$) as a function of concentration. The graph shows the effect of concentration and the increase in surface lipidic chain on the cytotoxicity of Caco-2 cells. At concentrations >0.30 mg/ml an increase in the length of the surface lipidic chain (from C_4 to C_{12}) of the dendrimer, resulted in an increase in cytotoxicity of the dendrimer-derived nanoparticles.

The increase in generation, from 5th to 6th, did not result in any increase in toxicity for dendrimers with C_4 and C_{10} surface lipidic chains (dendrimer I and II compared with dendrimer VI and V respectively. Comparing dendrimer III with VI, dendrimers with C_{12} surface lipidic chains, however, showed a slight increase in toxicity. The results were not significantly different, at a concentration of 0.8 mg/ml the viability of dendrimer III and VI was 42 ± 7 % and 34 ± 3 % respectively, but did indicate that an increase in generation lowered the cell viability.

Prior studies conducted, have only focused on charged dendrimers and surface modified dendrimers. The effect of the length of the surface modified lipidic chains on dendrimer derived nanoparticle toxicity has not been studied. Jevprasesphant *et al.* (2003) showed that PAMAM dendrimers with surface lauryl groups exhibited decreased toxicity, but in the same paper also showed that an increase in generation

showed some increase in toxicity. The results correlate with the present findings for dendrimers with C₁₂ surface lipidic chains where an increase in generation increased cytotoxicity. The results also suggest, that the length of lipidic chain on the dendrimers affects the dendrimer interaction with the cell resulting in a cytotoxic effect of Caco-2 cell, more specifically, an effect on the mitochondrial dehydrogenase activity (MTT assay) of Caco-2 cells. It is important to note that the MTT assay does not depend on severe cell damage to detect cytotoxicity and therefore the exact mechanism are not clear.

Other factors contributing to cytotoxicity is cationic charge and flexibility (Hoet *et al.* 2004). In chapter 2 it was stated that the larger dendrimers synthesised most probably did not have all surface amino groups coupled to lipidic chains, ideally only one NH₂ groups should be present in the core of each dendrimer. These uncoupled groups gives the dendrimers more cationic charges which could therefore contribute to the cytotoxicity. Lipidic chains on the surface of the dendrimers has shown to increase flexibility (Li *et al.* 2004). The increased flexibility enables more efficient membrane interaction (Hoet *et al.* 2004) and therefore the flexibility of the surface lipidic chains of the dendrimer-derived nanoparticles could be a contributing factor of toxicity.

To summarise, the toxicity of the lipidic dendrimer-derived nanoparticles was mainly dependent on the length of the surface lipidic chains and by the generation of the dendrimer used as a building block. A short carbon chain length (C₄ and C₁₀) did not show any toxicity while a C₁₂ lipidic chain showed increased toxicity. Toxicity observed *in vitro* towards one specific cell line can not be assumed to be synonymous with toxicity observed *in vivo*. Molecules *in vivo* interact with

biological fluids containing a variety of enzymes, proteins, growth factors etc. The content is likely to alter the toxicity of molecules. Furthermore cells from organ to organ vary in structure and in resistance towards molecules and will therefore also affect toxicity. It is also important to keep the dimensions in mind. The total area covered by the Caco-2 cells in a 96 well plate is 0.38 cm^2 ($r = 0.35\text{ cm}$) while the surface area of the epithelial lining in a rat small intestine is $\sim 157\text{ cm}^2$ ($r = 0.25\text{ cm}$, $h = 100\text{ cm}$), 400 times larger. As the IC_{50} value for dendrimer VI was $\sim 0.6\text{ mg/ml}$, approximately 250 mg should be given to a 150g rat in order to cause cytotoxicity, which is a very high dose. In the conducted animal experiments, described in chapter 5, a dose of 50 mg/kg of dendrimer III was given orally to animals, a concentration significantly lower than the toxic dose of 560mg calculated from its IC_{50} value ($\sim 0.7\text{ mg/ml}$).

4.3.2 Red blood cell lysis

Dendrimers taken up by the Peyer's patches after oral administration can ultimately reach the systemic circulation. The haematotoxicity of dendrimer-derived nanoparticles (derived from dendrimer I to VI) was therefore assessed.

The proposed theory for the toxic effect, is thought to be the interaction of the negatively charged cell surface and the positive charges on i.e. cationic PAMAM dendrimers (Carrefio-Gómez and Duncan 1997, Malik *et al.* 2000). It has also been shown that anionic and surface modified PAMAM dendrimers reduce the haemolytic activity. Here we assess the haemolytic activity of dendrimer-derived nanoparticles with lipidic surface groups (described in chapter 2). By surface modifying 5th and 6th

generation lysine based dendrimers with C₄, C₁₀ and C₁₂ lipidic chains, the effect of carbon chain length and generation on haemolysis was assessed.

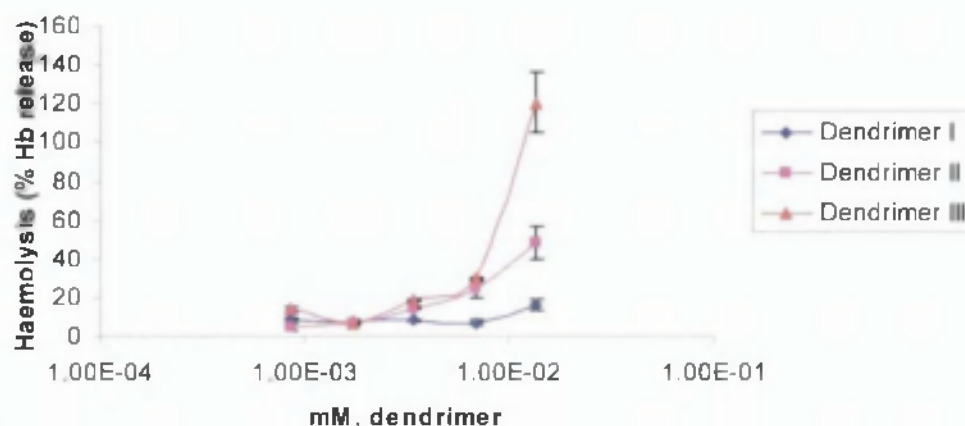


Figure 4.4. Dendrimer-induced haemolysis. The effect of dendrimer-derived nanoparticles concentration, derived from 5th generation dendrimers, on the haemolysis of red blood cells. An increase in lipidic chain length from C₄ to C₁₂ on the surface of the dendrimer resulted in an increase in the haemolytic activity of the dendrimer-derived nanoparticles. The molar concentrations used corresponded to a concentration that ranged between 0.005 mg/ml to 0.13mg/ml (n=5).

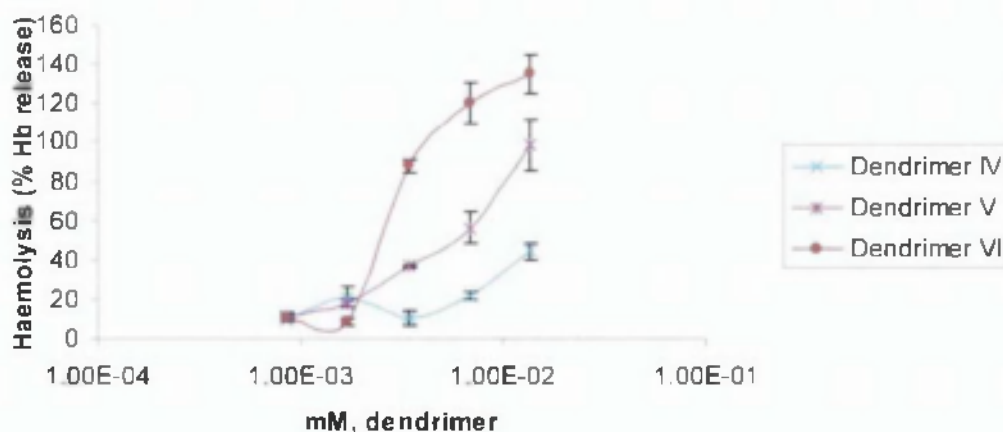


Figure 4.5. Dendrimer-induced haemolysis. The effect of dendrimer-derived nanoparticles concentration, derived from 6th generation dendrimers, on the haemolysis of red blood cells. An increase in lipidic chain length from C₄ to C₁₂ on the surface of the dendrimer resulted in an increase in the haemolytic activity of the dendrimer-derived nanoparticles. The molar concentrations used corresponded to a concentration that ranged between 0.01 mg/ml to 0.27 mg/ml (n=5).

Figure 4.4 shows the results for the 5th generation dendrimers and Figure 4.5 for the 6th generation dendrimer-derived nanoparticles. Haemolysis was only apparent in concentrations above 3.4×10^{-3} mM and 1.71×10^{-3} mM for the 5th and 6th generation dendrimer nanoparticles respectively. The results showed a generation related haemolytic activity when comparing dendrimer I with IV, II with V and III with VI (Figure 4.6). Increasing the carbon chain length from C₄ to C₁₂ resulted in an increase in the haemolytic activity of dendrimer-derived nanoparticles. The results showed a similar trend for both generations (Figure 4.4 and 4.5). The exact reason for the increase in haemolytic activity with an increase in carbon chain length is not apparent. However, considering the structure of red blood cells, it might very well be because of the fluid properties of the membrane. The red blood cell membrane contains equal amount of lipids, either phospholipids or neutral lipids, mostly unesterfied cholesterol, and proteins with the membrane phospholipids arranged asymmetrically into a lipid bilayer two molecules thick (Ballas and Krasnow 1980).

It can be suggested, that increasing the length of lipidic chains, on the surface of the dendrimers, from C₄ to C₁₂, increases the potential interaction between the red blood cell membrane and the dendrimers, resulting in increased haemolysis. Changes in the membrane structure results in morphological changes which results in increased cell death. SEM pictures of RBCs showed changes in morphology when incubated with all six dendrimers (Figure 4.7) and supported the proposed explanation. The erythrocytes developed into echinocytes which are characterized by their pointed surface structure.

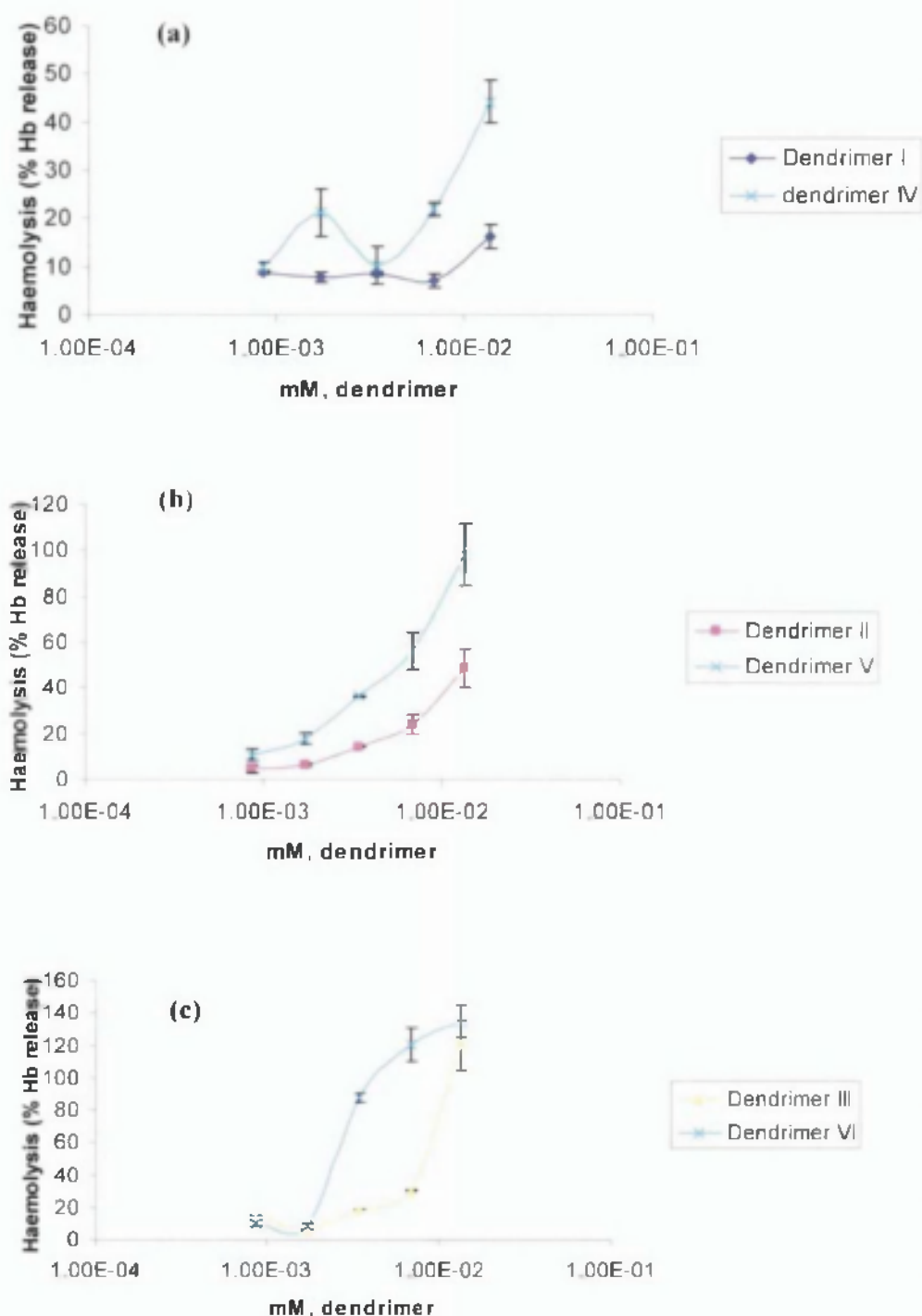
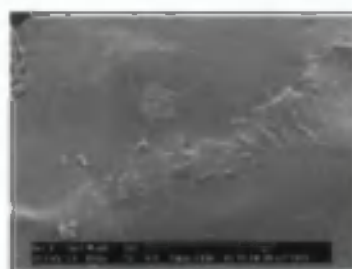
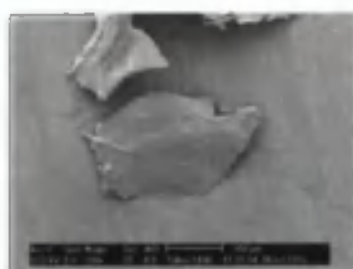
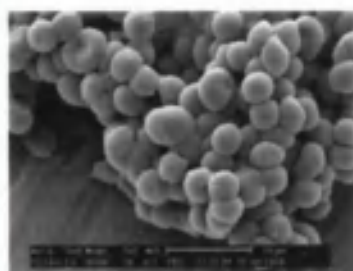


Figure 4.6 Dendrimer-induced haemolysis, showing the effect of dendrimer generation on the haemolysis of red blood cells. Dendrimer-derived nanoparticles derived from the 5th and 6th generation with either C₄ (a) C₁₀ (b) or C₁₂ (c) surface lipidic chains were compared. Regardless of the length of the lipidic chain, an increase in generation, from 5th to 6th resulted in an increased haemolytic activity and hence that particle size affects haemolysis (n=5).

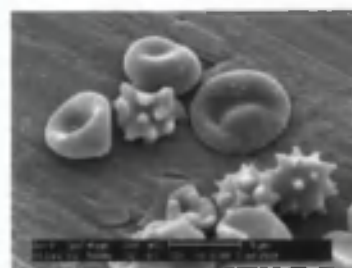
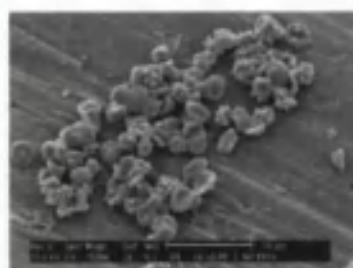
Triton X 100 (a)



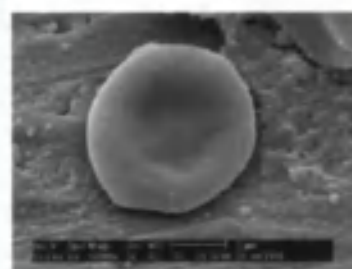
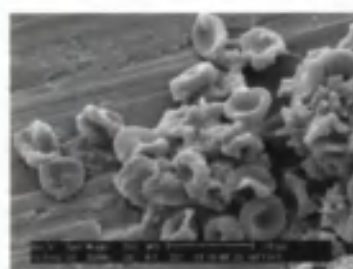
PBS (b)



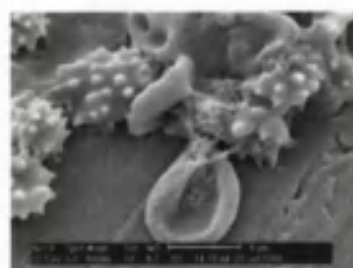
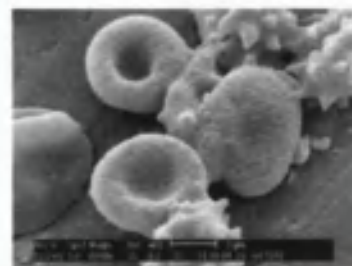
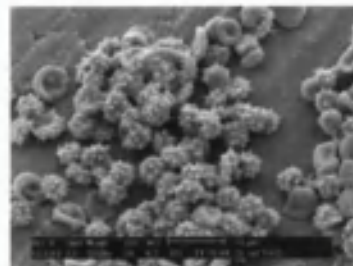
Dendrimer I (c)



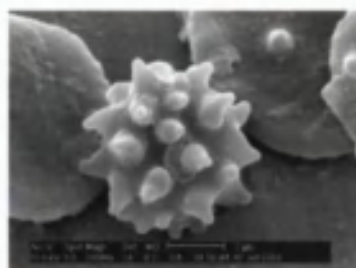
Dendrimer II (d)



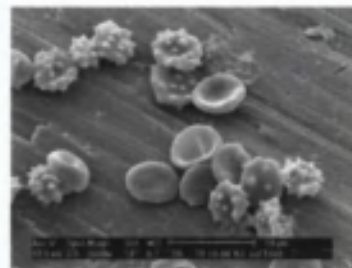
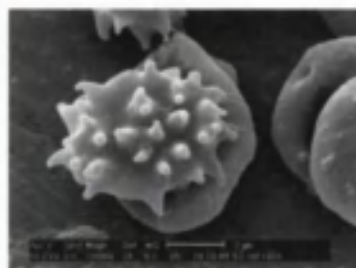
Dendrimer III (e)



Dendrimer IV (f)



Dendrimer V (g)



Dendrimer VI (h)

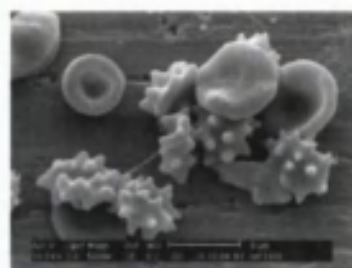
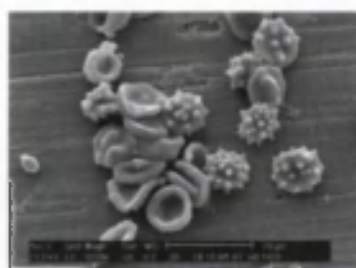


Figure 4.7. SEM of red blood cells, showing the morphological changes induced by the dendrimers (c, d, e, f, g, h), PBS, control (b) and Triton X-100, negative control showing 100 % lysis (a). Scanning electron micrographs shows (a) a complete solubilisation of red blood cells using Triton X-100 which was used as negative control where 100 % lysis occurred, (b) control in PBS, showing their classic biconcave morphology of red blood cells, (c to h) red blood cell morphology after incubation with dendrimer-derived nanoparticles, derived from dendrimers I to VI. Compared to control clear morphological changes were observed of red blood cells incubated with dendrimer-derived nanoparticles, with red blood cells developing into echinocytes characterised by their pointed structure. The concentration of dendrimer-derived nanoparticles used were 0.43 mg/ml for dendrimer I and II which corresponds to micrographs (c) and (d) respectively, 0.09 mg/ml for dendrimer III (e), 0.17 mg/ml for dendrimer IV (f) and V (g) and 0.009 mg/ml for dendrimer VI (h). The micrographs showed that even at low concentration of dendrimer-derived nanoparticles morphological changes of red blood cells occurred.

Malik *et al.* (2000) studied the haemolytic effect of cationic (MW 3256, 6909 and 14215) (> 1mg/ml) and a series of anionic PAMAM dendrimers (> 2 mg/ml). Interestingly the haemolytic activity for the nanoparticles from lipidic dendrimers I to VI was 30-50 μ g/ml significantly higher than for the PAMAM dendrimers. It is

important to note that although the molecular weight of the lipidic dendrimers ranged from 6290 to 19814, these were constructed into nanoparticles. This meant that the results were not directly comparable. The cell type (animal related variations), dendrimer type and diameter are factors likely to influence results. However the increase in haemolytic activity could be the effect of the strong interaction between the lipidic chains on the dendrimer and the red cell membrane as mentioned before. Furthermore, the red blood cells were suspended in PBS. It is possible that the salts present in the media could ameliorate the haemolytic effect by altering the surface nature of cationic PAMAM dendrimers, and result in comparatively low haemolytic activity. The potency of the lipidic interaction and the ultimate haemolysis caused, is not ideal. It should however also be considered that *in vivo* blood contains plasma with white blood cells, platelets, proteins etc. The composition and nature of whole blood is likely to alter the haemolytic activity of the dendrimer and dendrimer-derived nanoparticles. This is an area that still needs to be studied.

The dendrimer were synthesised with oral administration in mind, and further targeting to the M-cells of the Peyer's patches. The haemolytic concentration *in vivo* may alter from the concentration found in this chapter. However, the amount of dendrimer which will ultimately be translocated through to the systemic circulation, should not exceed the haemolytic concentration *in vivo*. The uptake and translocation of the dendrimer will be discussed in chapter 5.

The toxicity of dendrimer-derived nanoparticles towards an epithelial cell line has been described and assessed. The toxicity of dendrimer-derived nanoparticles *in vivo*, however, is largely influenced by their stability in biological media in which they are transported. In addition, the stability of the dendrimer-derived nanoparticles will

affect dendrimer uptake. In the next two sections the stability of the dendrimers in relevant biological media will be discussed.

4.3.3 The stability of dendrimer-derived nanoparticles in simulated intestinal and gastric fluid

The physical stability of dendrimers prior to and during *in vivo* uptake is an area which has so far not been studied in great detail. In order to improve the understanding of the mechanisms involved, the stability of the dendrimer-derived nanoparticles, derived from dendrimer III was assessed in simulated intestinal and stomach fluid containing pancreatin and pepsin respectively. The suspension of dendrimer-derived nanoparticles were incubated with intestinal and stomach fluid inside the dialysis bag. Using a dialysis membrane with an MWCO of 3500, the cut off being significantly smaller than the molecular weight of a single dendrimer (MW 9881) and dendrimer-derived nanoparticles (Approximately MW 69167, if average diameter ~25nm), only break down products of the dendrimer will pass through the membrane because of the increased solubility of these fractions. No interaction of the lipids or cationic molecules with the cellulose snake skin dialysis membranes were possible (Company leaflet) unlike in the case of regenerated cellulose membranes, where some of the $-CH_2OH$ groups are oxidised to $-COOH$ in air (Canas *et al.*, 2002). This gives a weak anionic charge to these membranes which can reduce the passage of cationic molecules and assist their adsorption.

Using a radioactive carbon labelled dendrimer (III), with the label attached on the lipidic chains on the surface of the dendrimer, dendrimer-derived nanoparticles were formulated with a concentration of 1.71 mg/ml and incubated with simulated

intestinal and stomach fluid (Figure 4.8). The pH of the solutions was 1.2 and 7.5 respectively.

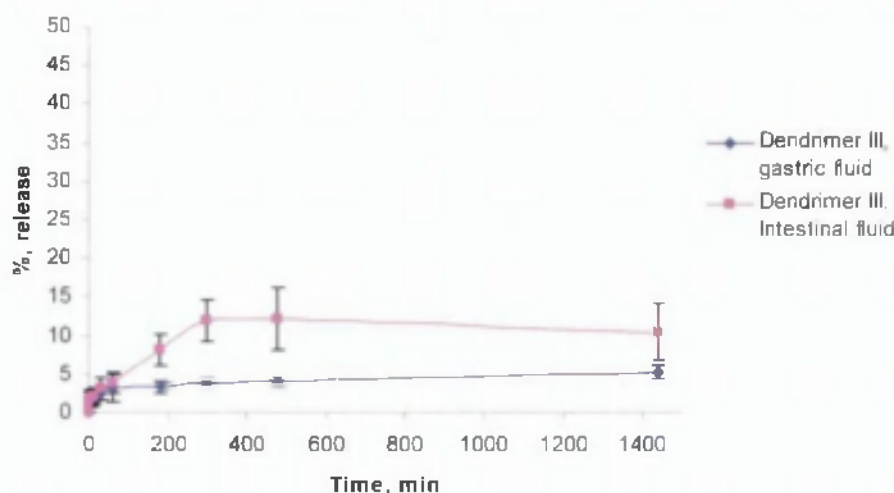


Figure 4.8 Showing the dialysis (MWCO 3500) of dendrimer III (C_{12} surface lipidic chains) incubated with simulated gastric and intestinal fluid. The dendrimer shows a very low release of the dendrimer, indicating that the dendrimer is stable towards enzymatic degradation and that the radioactive label on the lipidic chain is not detached when it comes into contact with enzymes present in the stomach and intestine ($n=3$).

The results showed that in simulated gastric or intestinal fluid, ~7% and 12 % respectively of the radioactive dendrimer (III), was transported through the membrane over a period of 24 h. This meant that the radiolabel attached onto the lipidic chain of the dendrimer was stable in gastric fluid, because no accumulation of the radioactivity was observed in the outer dialysis chamber. If the radiolabel was detached, an accumulation would be expected within a few hours. The results however suggested, that the dendrimer underwent a slow break down over 24 hours. Whether the lipidic chain or a larger segment of the dendrimer was detached is not apparent. The results do suggest, that dendrimer-derived nanoparticles, over a time period corresponding to the stomach and intestinal transition time of 25min and 3-8h respectively, have only 3% and 8 to 12 % transported through the membrane. The rate at which the dendrimer was broken down, should therefore not to any great

extent, affect the dendrimer uptake. In both intestinal and stomach fluid, a sharp initial rise was observed at the lower time points, suggesting that the lipidic chains facing out towards the solution was more prone to degradation. The packing of the dendrimer into nanoparticles ensured, that large proportions of the lipidic chains were buried inside the dendrimer nanoparticles and provided physical protection against enzymatic degradation. This however, would only be possible if the dendrimer-derived nanoparticles did not disassociate in the respective media. Several other mechanisms for the protection were possible i.e. adsorption/interaction of the enzymes onto dendrimer nanoparticles or onto dendrimers after disassociated from the nanoparticles. In the following section, the physical stability of dendrimer-aggregates in biological fluids is assessed.

4.3.4 Effect of simulated intestinal and stomach fluid on apparent particle size of dendrimer-derived nanoparticles degradation

Amphiphillic dendrons have shown to form aggregates when they come in contact with cell culture media (Singh et al 2003), posing problems for interpretation of cellular interaction. Nanoparticles derived from dendrimer II and III (1.71 mg/ml) were incubated with purified intestinal and stomach fluid. The apparent particle size was measured by PCS over 180 min. The pH of the intestinal and stomach fluid was 6.5 and 4.8 respectively. The results showed that over a 3h period the dendrimer were stable in intestinal fluid (Figure 4.9) whereas on incubation in stomach fluid (Figure 4.10) there was an immediate increase in apparent particle diameter (~2000-3000nm)

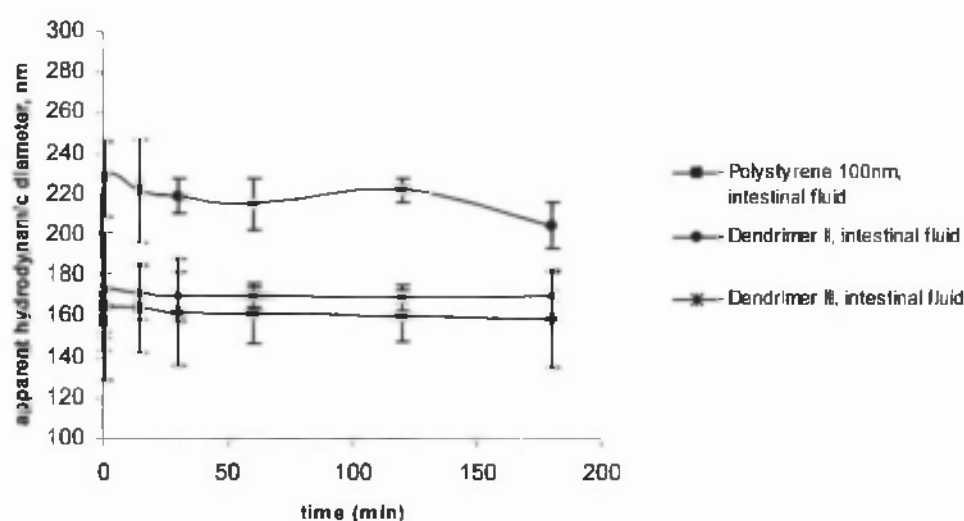


Figure 4.9. A plot showing the effect over a 3 h time period of purified stomach fluid on apparent diameter of aggregates prepared from dendrimers II and III. Compared to polystyrene nanoparticles (100nm), the dendrimers showed an immediate and significant increase in apparent particle size (~2000-3000nm) (n=3).

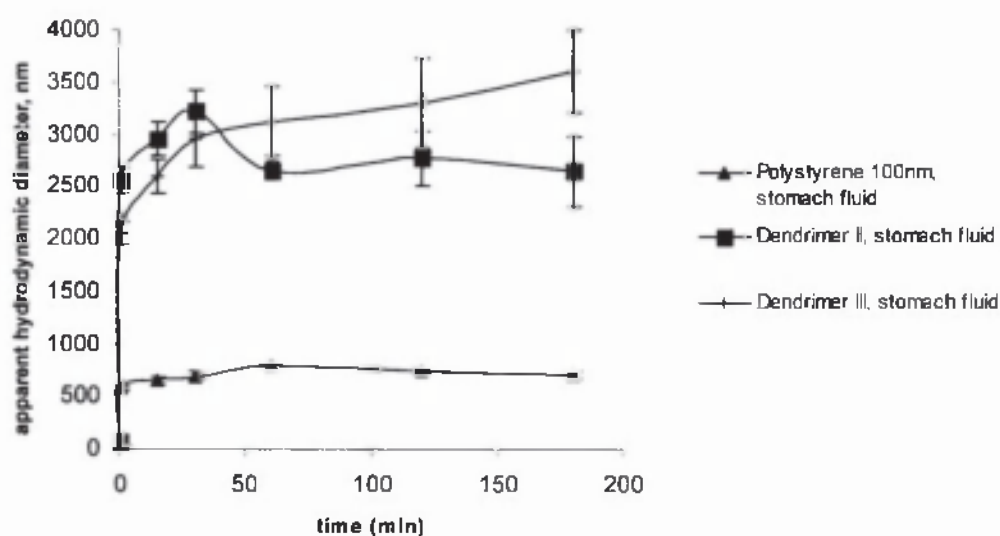


Figure 4.10. A plot showing the effect over a 3 h time period of purified intestinal fluid on apparent diameter of aggregates prepared from dendrimers II and III. Compared to polystyrene nanoparticles (100nm), the dendrimers showed no significant increase in apparent particle size was observed in intestinal fluid (~220nm) (n=3).

The SEM pictures of dendrimer-derived nanoparticles in purified intestinal and stomach fluid (Figure 4.11) suggests, that aggregation of the system does occur. Regardless of the biological fluid in which the dendrimer derived nanoparticles were

incubated, there was a significant increase in size from the initial 25nm. The particles were clustered together regardless of the biological fluid used, but with the dendrimer-derived nanoparticles more clearly defined in intestinal fluid. The dendrimer-derived nanoparticles in stomach fluid were hidden among debris, suggesting interaction between dendrimer-derived nanoparticles and the constituents of stomach fluid to a greater extent than in intestinal fluid. The results suggest, that the protection against enzymatic degradation is mainly governed by adsorption/interaction of constituents in intestinal and stomach fluid.

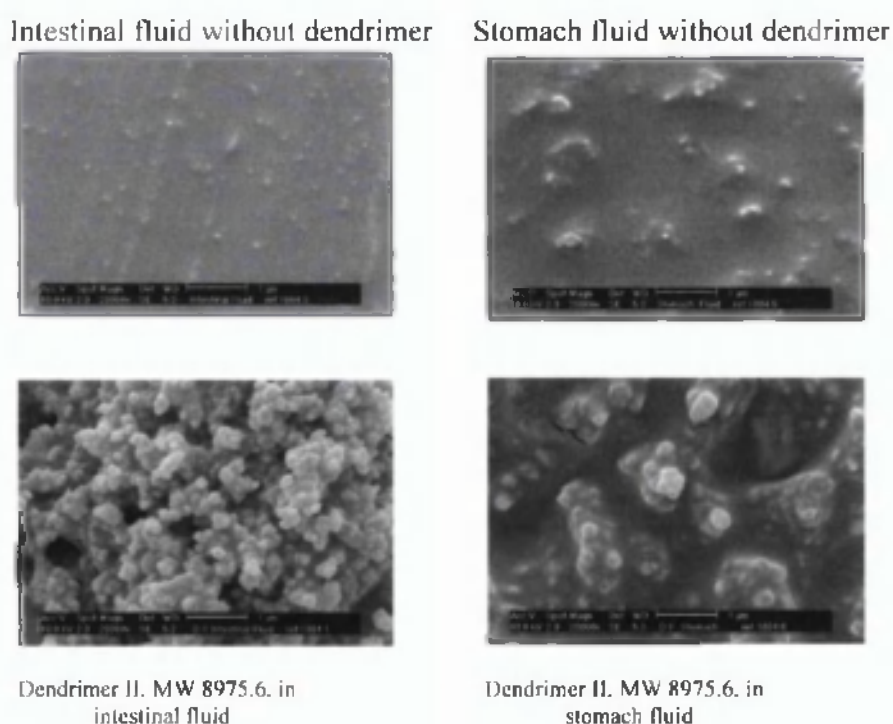


Figure 4.11. SEM pictures of purified intestinal and stomach fluid with (lower Figures) and without (upper Figures) dendrimer II-derived nanoparticles (1.71 mg/ml) after 3 h incubation. Dendrimer-derived nanoparticles in stomach fluid were hidden among debris suggesting interaction with constituent of stomach fluid, whereas in intestinal fluid the particles were more clearly defined, symptomatic of less interaction.

4.4 Conclusion

The toxicity of dendrimer-derived nanoparticles, in the limited toxicological evaluation presented here, was determined by the length of lipidic chains and generation of dendrimers used. Dendrimer-derived nanoparticles from dendrimers with C₁₂ surface lipidic chains attached showed a comparatively higher toxicity in Caco-2 cells and towards red blood cells than dendrimer nanoparticles derived from dendrimers with C₄ and C₁₀ surface lipidic chains. The longer lipidic chain seemed to interact more strongly with the cell membranes and hence cause greater haemolysis or cytotoxicity. The dendrimer-derived nanoparticles were stable against degradation in biological fluids. The aggregation of the system in the stomach and intestinal fluid was likely to contribute to the protection, of the dendrimers, against degradation but also likely to affect the absorption of these systems *in vivo*. The uptake of dendrimer-derived nanoparticles after oral administration will be discussed in the following chapter.

Chapter five

In vivo studies – oral uptake and histology

5.1 Introduction

In this chapter the biodistribution of dendrimer-derived nanoparticles after gavage was assessed. Study of the factors affecting the oral uptake and translocation of particles has been an area of great interest and much discussion. It was often assumed that the mammalian gastrointestinal tract was a barrier impenetrable to the transport of particulate matter, and the debate has mainly evolved around the acceptance or otherwise whether particulate uptake was a process which reliably occurred. The work carried out by several laboratories (LeFevre *et al.* 1978a.b, 1980, 1989., Eldridge *et al.* 1990, Rolland 1993, Jani *et al.* 1989, 1990, 1992) showed uptake of nanospheres through specialized regions of the GI tract , the Peyer's patches (PP). It is now widely accepted that particulate matter can be taken up by Peyer's patches and the areas surrounding them. Uptake of inert particles has been shown to occur transcellularly through normal enterocytes and PP via M-cells and to a minor extent

across para-cellular pathways (Aprahamian *et al.* 1987). PP differentiate between the type and size of particles. Altering particle characteristics such as size (Hillyer and Albrecht 2001), surface charge (Jani *et al.* 1989), surface attachment of ligands (Hussain and Florence 1998, Hussain *et al.* 1997) or coating with poloxamers (Hillery *et al.* 1994) offers possibilities for targeting specific regions of the gastro intestinal tract such as PP.

Fluorescent polystyrene nanoparticles have been widely used in order to determine particulate uptake as they have been easy to visualize and quantify through gel permeation chromatography. A wide size range of polystyrene microspheres has been studied, and a size dependent uptake and translocation was observed (Jani *et al.* 1990). 50nm particles were translocated to a greater extent than 100nm particles, whereas 300nm particles were absent from the blood. This has led to the suggestions that an optimal size exists where particles are translocated to a greater extent. Furthermore Szentkuti (1997) showed that the smaller the particle diameter the faster they could permeate the mucus to reach the colonic enterocytes. 14nm particles permeated within 2 min, 415nm took 30 min and 1000nm particles were unable to penetrate this barrier. A quantitative study using polystyrene particles less than 50nm in diameter has still not been conducted and therefore it is still not apparent if an optimal size exists.

One of the major issues of polystyrene particles, is their non-biodegradable nature. In search of a carrier that is biodegradable and nanoscopic in size, dendrimers has been synthesized. The biodegradability of lysine based dendrimers is an area which needs further examination. Dendrimers serve several purposes. Besides being nanoscopic, they also provide possibilities of surface modification. Therefore dendrimers are a

valuable alternative to polystyrene particles and provides immense possibilities and expectations.

5.1.2 Oral uptake of dendrimers

Even though many *in vitro* experiments have been conducted using dendrimers, so far only two papers have been published on oral uptake and translocation of dendrimers (Sakthivel *et al.* 1999; Florence *et al.* 2000). Sakthivel *et al.* (1999) and Florence *et al.* (2000) utilized a 4th generation lysine based dendrimer, with 16 C₁₂ surface alkyl chains, and a molecular weight of 6300 and a diameter of 2.5nm for the oral uptake studies. The dendrimer [Gly(Lys)₁₅(C₁₂)₁₆] was given as a oral aqueous solution and showed the following biodistribution after oral administration to fasted female Sprague-Dawley rats (14mg/kg and 28mg/kg) (Table 5.1).

Table 5.1. the biodistribution of a lipidic dendrimer [Gly(Lys)₁₅(C₁₂)₁₆] after oral administration of 14mg/kg and 28mg/kg in the small intestine, large intestine, blood, liver, spleen and kidneys.

	Small intestine	Peyer's patches small intestine	Large intestine	Peyer's patches large intestine	Blood	Liver	Spleen	Kidneys
14mg/kg, 6 h	15%		5%		3%	1.5%	0.1%	0.5%
28mg/kg, 3 h	3.8 %	1.5 %						
28mg/kg, 12 h	0.3 %	0.05 %	3.8 %	0.3 %				

(Florence *et al.* 2000).

Administering 14mg/kg showed that more than 20% of the administered dose was recovered from the stomach, small intestine and large intestine. However this figure

represents the material which was absorbed, adsorbed or otherwise associated with the tissues. After 24 h less than 1% of the administered dose was recovered from all the organs studied, indicating excretion of unabsorbed dendrimer or clearance of the absorbed dendrimer. The levels of uptake and translocation of the dendrimer were compared to results obtained studies with polystyrene nanoparticles (Figure 5.1)

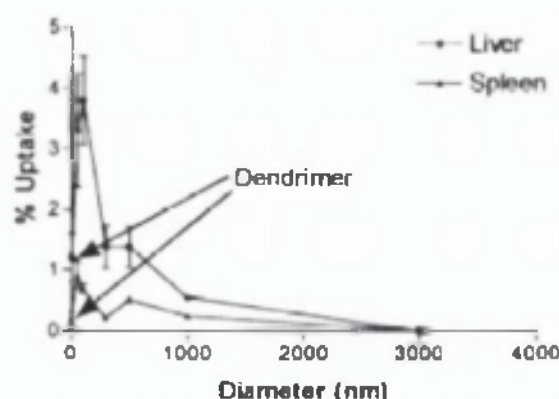


Figure 5.1. Uptake of orally administered dendrimer and polystyrene latex in the liver and spleen as a percentage of administered dose as a function of particles size. The dose of dendrimer was 14 mg/kg (single dose) whereas, polystyrene latex particle were 1.25 mg/kg for 10 days (Taken from Florence et al 2000).

The uptake into and translocation to the liver and spleen of the dendrimer was less than that achieved by the 50nm polystyrene nanoparticles and with the same trend was evident for the cumulative uptake in liver, spleen, kidney and blood. The results underlined the fact that there may be an optimum size for particle uptake, taking into account the fact that the dendrimers and the polystyrene particles are different chemically.

In this chapter, oral uptake and translocation of 5th generation lysine based dendrimers with 32 surface alkyl groups (C_{12}) is evaluated, but not as such. The dendrimers were formulated into nanoparticles with a particle diameter of 23nm. The

assembly of dendrimers will be an interesting comparison to the previous dendrimer study described above, but also to the polystyrene nanoparticles experiments, as an optimum size for particulate uptake after oral administration might be determined.

5.2 Materials and Methods

5.2.1 Materials

Dendrimer III, (*MW 9881.2*) was as described in chapter 2. Female Sprague Dawley rats (~150g) were purchased from Harlan, UK, LTD. Phenobarbital sodium (Sagatal) was obtained from Rhone Mérieux Ltd, Harlow, Essex, isoamyl alcohol, hydrogen peroxide (30.4 %) from Sigma UK; glacial acetic acid from BDH, Leicestershire, UK. Lithium-heparin tubes (4.5 ml, 75 x 13 mm) were purchased from Sarstedt, Germany, Optiphase 'Safe' from Wallac, Finland (formulated and produced by Fisher Chemicals, UK). Solusol was obtained from National Diagnostics, Hessle, Hull, UK. A Polytron homogeniser, PT 3000, Kinematica AG, UK was utilised for tissue homogenising. Phosphate buffered formalin fixative was obtained from Sigma, UK.

5.2.2 Methods

5.2.2.1 Formulation of dendrimer-derived nanoparticles

Dendrimer-derived nanoparticles, derived from dendrimer III (Figure 5.2) were formulated as described in section 3.2.1. Method 2. The average particle size was 25 nm, which was confirmed using photon correlation spectrometry. The particles were formulated so that administered material in 0.5 ml corresponded to 50 mg/kg for each animal.

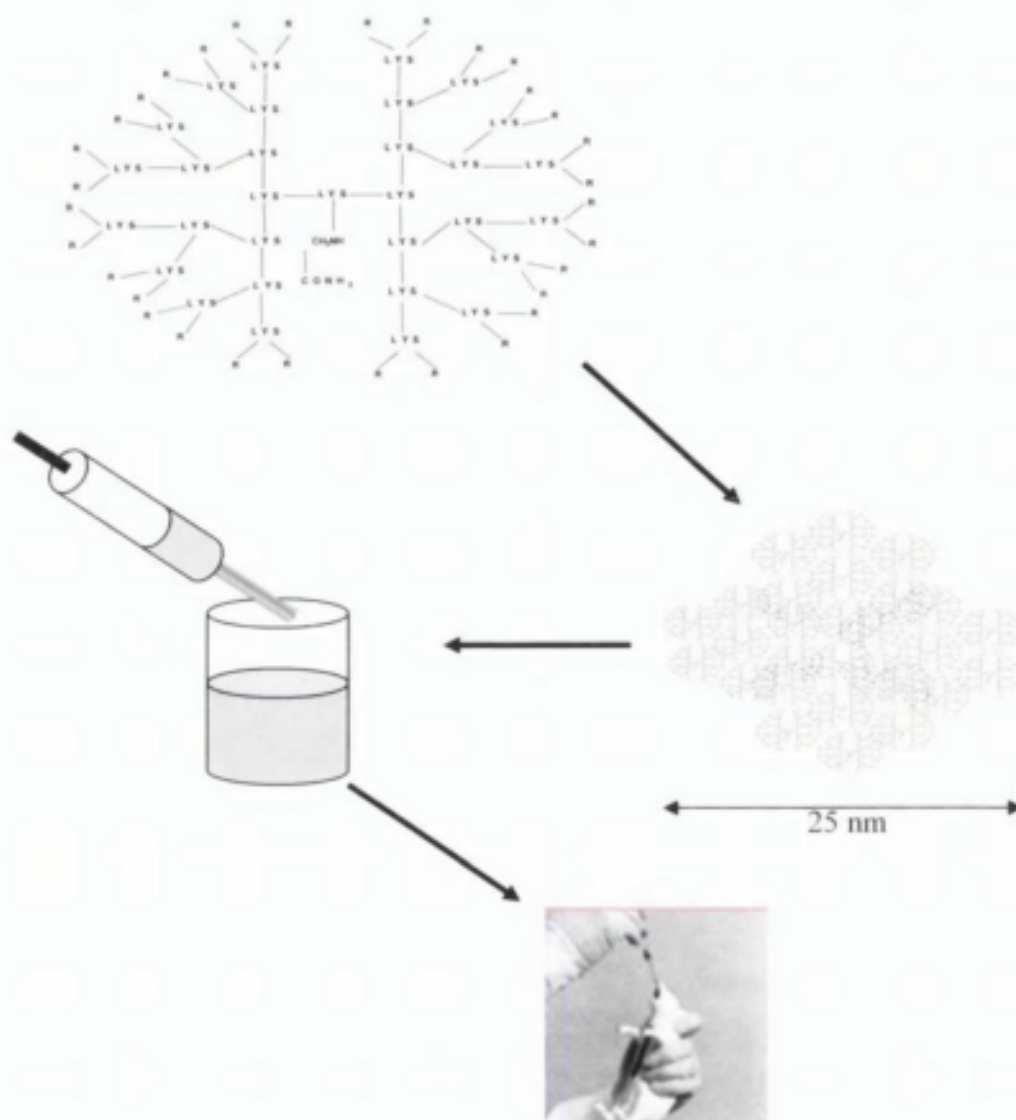


Figure 5.2. A schematic representation of the experimental process. Dendrimer-derived nanoparticles were formulated from dendrimer III (R represents dodecanoic acid) and then given by gavage to female Sprague Dawley rats using a blunt tipped feeding needle.

5.2.2.2 Oral uptake studies

The dendrimer-derived nanoparticles, derived from dendrimer III were administered orally to fasted (overnight) female Sprague Dawley rats (150g) (dose: 50mg/kg, 0.5 ml, n =3), using a gavage needle inserted into the stomach. At time points 1, 3 and 24 h post dosing, the animals were killed by interperitoneal injection of an overdose of anesthetic (Euthatal, phenobarbital sodium). A blood sample was taken immediately from the abdominal aorta and anticoagulated with lithium-heparin. The stomach, small intestine (without Peyer's patches), small intestinal Peyer's patches, large intestine, mesenteric lymph nodes, liver, spleen, heart and kidneys were then removed (Figure 5.3).

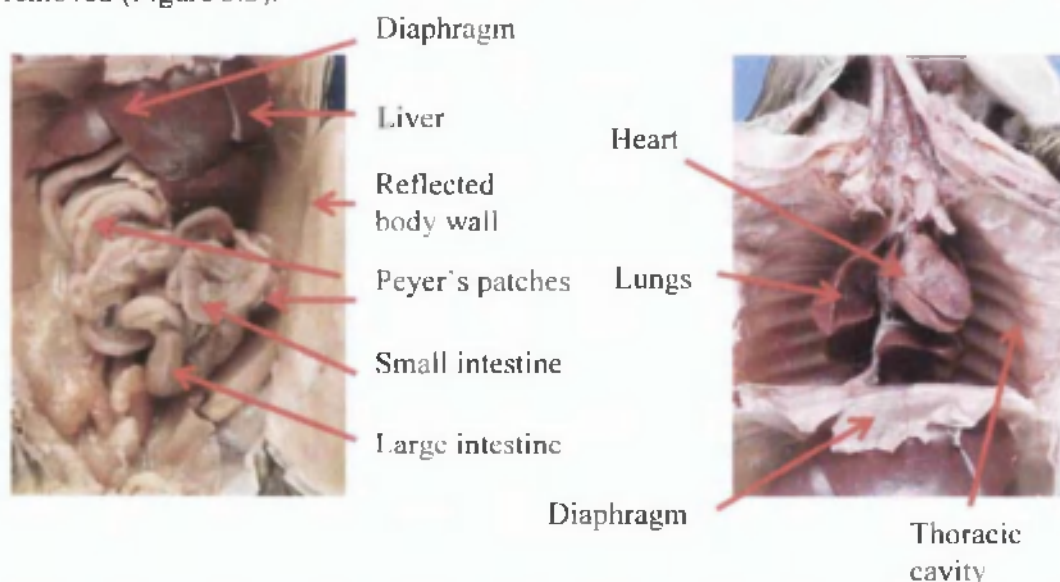


Figure 5.3. The major organs of the abdomen and thorax of the rat.

The right hind leg was removed. The femur was separated from the surrounding muscle and cleaned of extraneous tissue. The proximal and distal femoral epiphyses were removed using bone forceps and the contained bonemarrow flushed into 4 ml of double distilled deionised water using a needle and a 1.0 ml disposable syringe and anticoagulated with lithium-heparin. All tissues and organs were weighed on removal

and homogenised. An aliquot of homogenate was solubilised in tissue solubiliser (Solusol, 3 ml) and digested at 60°C overnight. The samples were then decolorized using 0.4 ml 30 % H₂O₂ and the antioxidant isoamyl alcohol (0.2 ml) was added and incubated at 50°C for 30 min. Glacial acetic acid (200 µl) was then added to eliminate chemiluminescence along with 10 ml scintillation fluid (Optiphase). The samples were kept in a dark cold room for 72 h and analysed for radioactivity.

5.2.2.3 Histology

Dendrimer-derived nanoparticles as above were administered orally to fasted female Sprague Dawley rats (150g) (dose: 50mg/kg, 0.5 ml, n = 5, control n =3) and control animals were administered double distilled deionised water using a gavage needle inserted into the stomach. The body weights of the animals were monitored over a 32 h period to assess body weight changes after the administration of dendrimers. After 32 h the animals were killed using CO₂ overdose and the stomach, small intestine with Peyer's patches, large intestine, mesenteric lymph nodes, liver, spleen, heart, kidneys (left and right), jejunum and colon removed and weighed. The tissues were fixed in 10.5 % phosphate buffered formalin fixative for 14 days. The stomach was cut longitudinally, the contents removed by gentle washing with water and the open stomach pinned onto a piece of carton to prevent contraction of the tissues. Sections (3–4 µm) of the tissues were prepared and stained with hematoxylin and eosin (H & E) for histological examination using light microscopy.

5.3 Results and discussion

5.3.1 Gastro intestinal organs

The amount of dendrimer recovered from the stomach, small intestine (without the Peyer's patches), Peyer's patches and the large intestine at different time points is given in Figure 5.4.

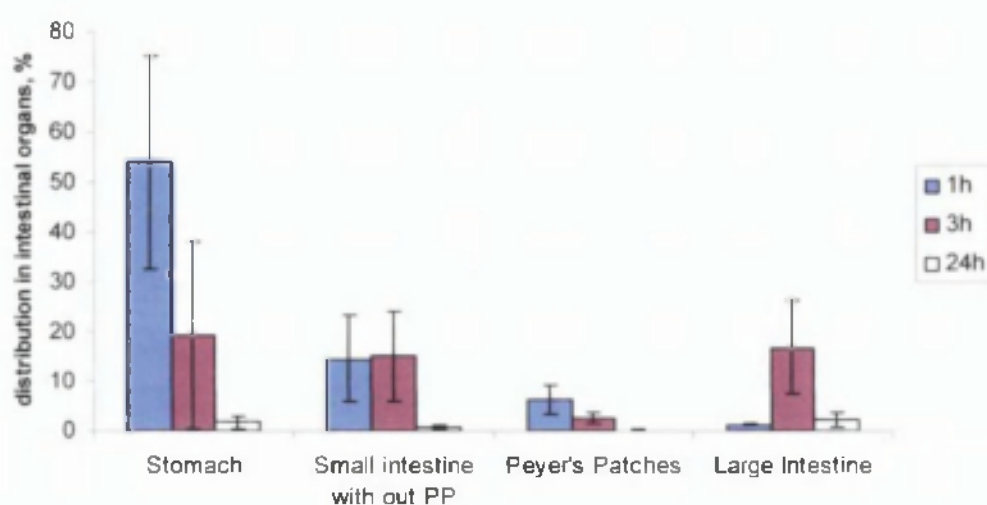


Figure 5.4. The percentage of dose in four locations in the GI tract of the dendrimer-derived nanoparticles administered as a single dose of 50 mg/kg at 1, 3 and 24 h. The results show the flow through the gastro-intestinal tract from the stomach to the large intestine. The Peyer's patches represent a very small area when compared to that of enterocytes. The data represent values for absorbed, adsorbed and dendrimer which is otherwise present in the areas.

The tissues were kept unwashed in order to follow the natural transit through the gastro-intestinal tract, providing information about accumulation of dendrimers in various parts of the gut at different time points, more specifically the Peyer's patches. The reasoning behind not washing the tissue was that previous studies have shown uptake through Peyer's patches (Saktivel *et al.* 1999; Florence *et al.* 2000) but at relatively low levels. As a comparatively larger amount of dendrimer was recovered

in the PP, the dendrimer-derived nanoparticles specifically targeted this area of the small intestine.

The results showed, as expected, a flow of any unabsorbed dendrimer from the stomach and through the intestine over a 24 h period. In the first hours after oral administration the majority of the dendrimer-derived nanoparticles were present in the stomach ($\sim 54 \% \pm 21 \%$) with $15 \pm 9\%$ present in the small intestine without PP and $6 \pm 3 \%$ in the PP. Of the total amount of dendrimer-derived nanoparticles present in the entire small intestine (21 %) approximately 29 % was recovered in the areas surrounding the PP. This is a substantial amount considering that the Peyer's patches only make up a very small part of the small intestine. The surface area of the small intestine is $\sim 200 \text{ m}^2$. The length of the small intestine is approximately 100 cm and containing between 10-15 Peyer's patches with a longitudinal size of $\sim 0.4 \text{ cm/PP}$. As a rough calculation this means that the area of the PP is less than a 16^{th} of the total area of the small intestine.

After 3 h, $19 \pm 9 \%$ of the dendrimer-derived nanoparticles were present in the stomach with 18 % in the small intestine. Again a substantial amount of the dendrimer-derived nanoparticles present in the small intestine were located around the PP ($2.6 \pm 1 \%$). The results indicating that the dendrimer-derived nanoparticles were taken up relatively more effectively by PP. In the time interval from 1 to 3 h the dendrimer-derived nanoparticles in the small intestine without the PP remained constant around 15-18 %, whereas the amount in the areas of the PP decreased from $6 \% \pm 3 \%$ to $2.6 \pm 1 \%$ suggesting a comparatively larger absorption from PP or simply a saturation of the uptake process (Sternson 1987).

There was a steady flow of dendrimer derived nanoparticles down through the intestinal organs with 20 % of the dendrimer-derived nanoparticles recovered from the large intestine, 18 % from the small intestine and 19 ± 9 % from the stomach after 3 h. Considering that the large intestine showed a marked increase in the amount of dendrimer-derived nanoparticles recovered from 1 to 3 h and that the small intestine showed an almost constant level of dendrimer derived nanoparticles in the same time period, the movement through the intestine was quite rapid (1 to 3h). With most of the PP present in the lower part of the small intestine, the area considered most potent in regard to uptake of nanoparticles, translocation of dendrimer-derived nanoparticles must be at peak during this time period, especially given that 14nm polystyrene nanoparticles were able to penetrate the mucus in 2 min (Szantkuti 1997). Dendrimer-derived nanoparticles were larger in diameter, at 23nm, but with a flexible surface (lipidic chains), and therefore the permeability rate through the mucosa would be expected to be fairly similar. Physically this would mean that as soon as the dendrimer-derived nanoparticles are at the target area (PP) they would be translocated within minutes.

After 24 h most of the unabsorbed dendrimer-derived nanoparticles were excreted through the faeces with only $2 \% \pm 1$ % still present in the stomach, 0.7 ± 0.5 % in the small intestine without the PP, 0.1 % present in the PP and 2.3 ± 1 % present in the large intestine.

The stomach (Figure 5.5), ileum (Figure 5.6) and Peyer's patches (Figure 5.7) from control and dendrimer-treated animals were examined histologically. There was no evidence of dendrimer induced changes in tissues or in the weight of the animals (Figure 5.8) at dendrimer concentrations of 50 mg/kg. The results demonstrated that

dendrimers taken up in the stomach, small intestine and Peyer's patches did not show any overt toxicity.

Comparing the *in vivo* and *in vitro* (chapter 4) toxicity of dendrimer III, it is apparent that a toxic effect *in vitro* does not necessarily result in a toxic response *in vivo*. The concentration of cells, dendrimer and biological fluids provide a much more complex environment by which toxicity can be ameliorated.

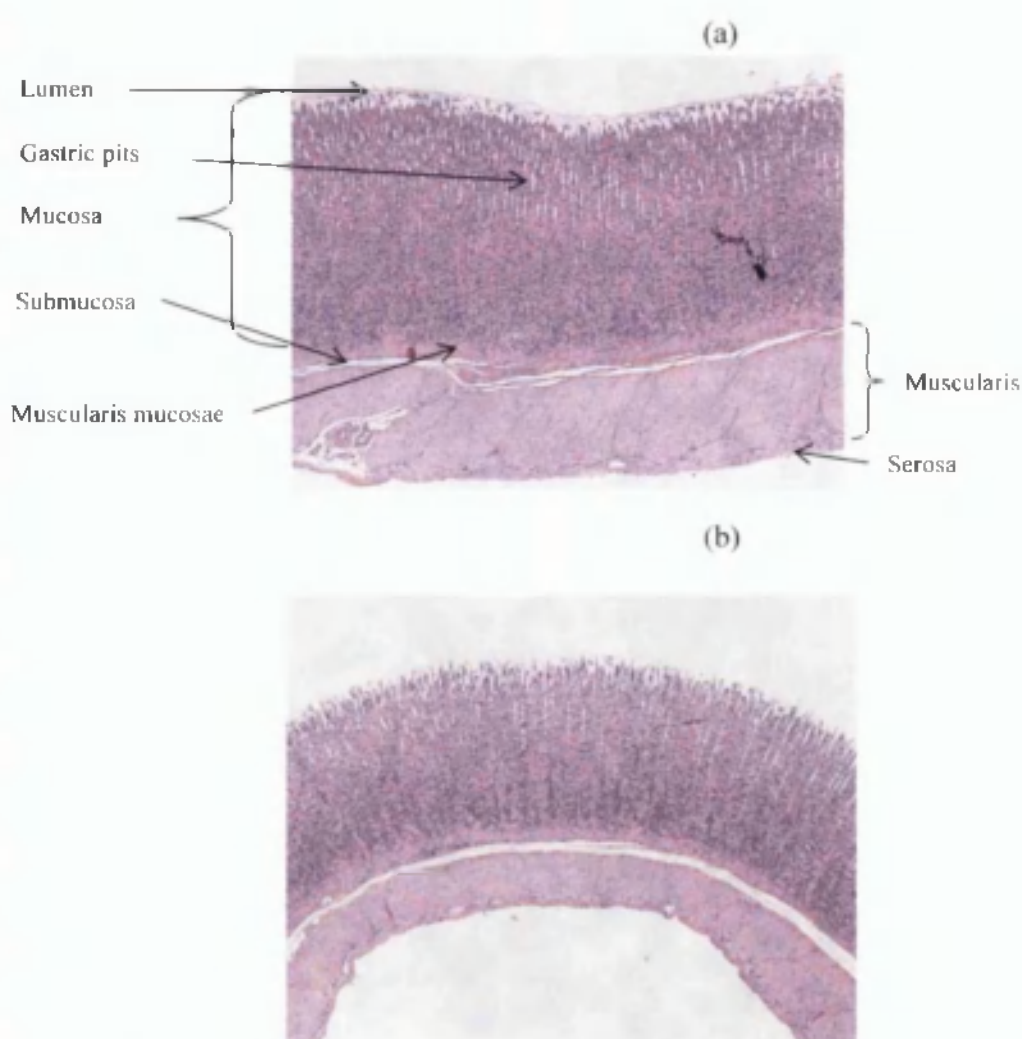


Figure 5.5. Images of sections (3-4 μm) of the rat stomach. Fixed in 10.5 % Phosphate buffered formalin and H &E stained (X 100). (a) represents dendrimer treated (32 h) and (b) control. Labels on (a) also refer to (b). No differences were observed between control and treated animals.

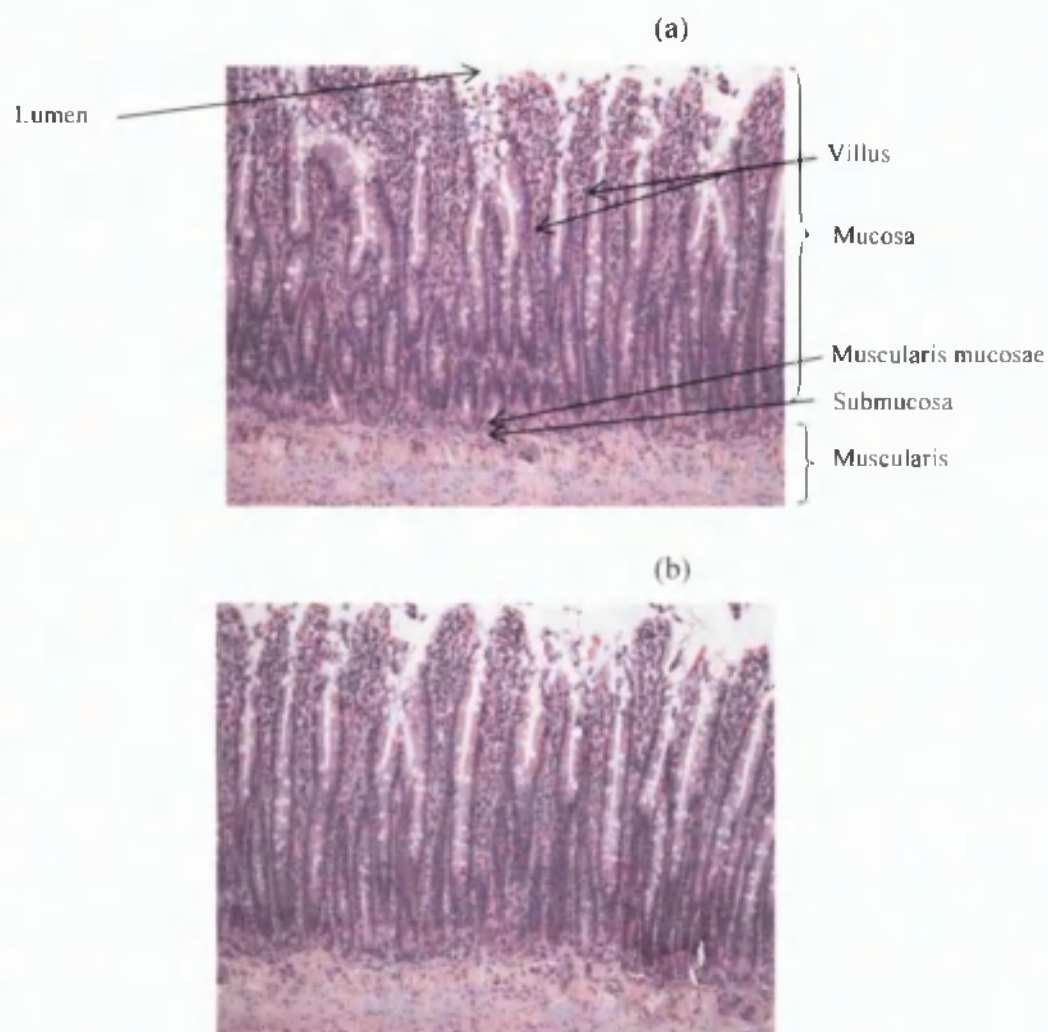


Figure 5.6. Images of sections (3-4 μm) of the rat ileum, fixed in 10.5 % phosphate buffered formalin and H &E stained (X 100). (a) represents dendrimer treated (32 h) and (b) control. Labels on (a) also refer to (b). No differences were observed between control and treated animals.

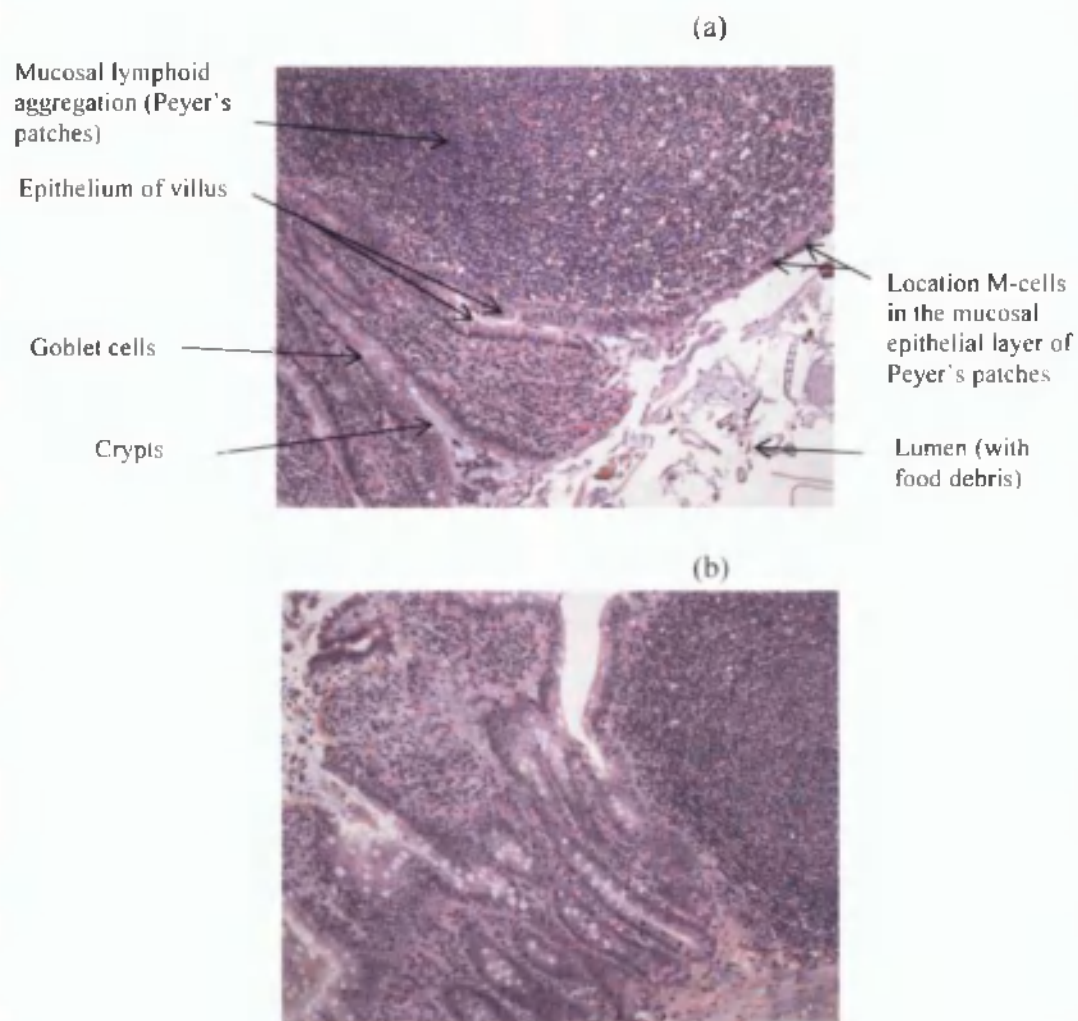


Figure 5.7. Images of sections (3–4 μm) of the rat Peyer's patches, fixed in 10.5 % Phosphate buffered formalin and H & E stained (X 100). (a) represents dendrimer treated (32 h) and (b) control. No differences were observed between control and treated animals.

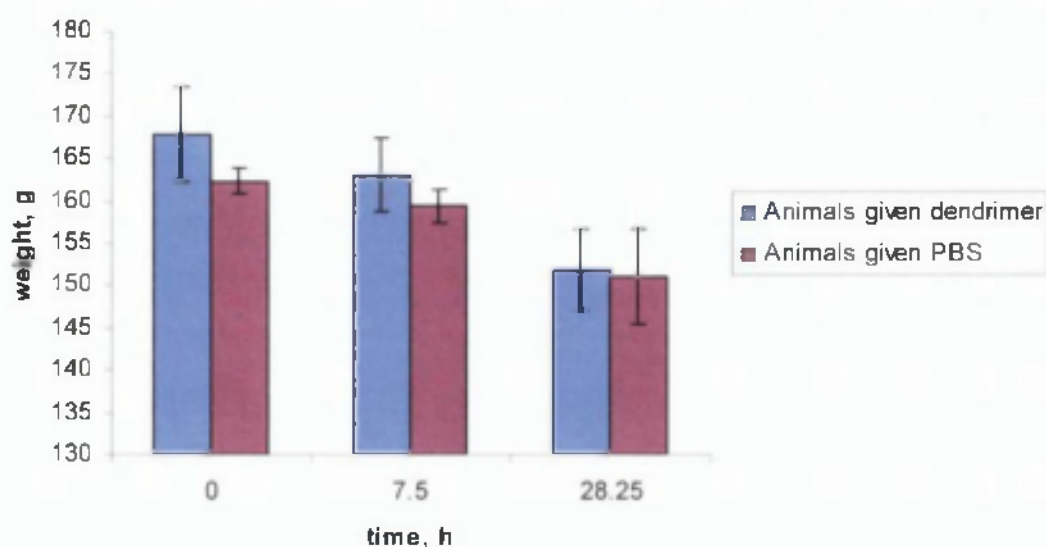


Figure 5.8. Weight of animals female Sprague-Dawley rats (n=5, control n =3) dosed with 50 mg/ml dendrimer-derived nanoparticles (at 0 h). Weight monitored over 28 h. Approximately 7.5 h after dosing the animals were given food. The weight loss was observed for both control and dendrimer treated and therefore was not due to the dendrimer-derived nanoparticles, but rather a disturbance in their eating habits.

5.3.2 Major organs of the abdominal and thoracic cavities

When estimating uptake of dendrimers, most emphasis is given to the major organs of the abdominal and thoracic cavities such as liver, spleen, kidney, heart, mesenteric lymphnodes, bone marrow and blood as this gives a true indication of the extent of translocation of particles. The amount of dendrimer-derived nanoparticles recovered in these organs is given in Figure 5.9 and 5.10.

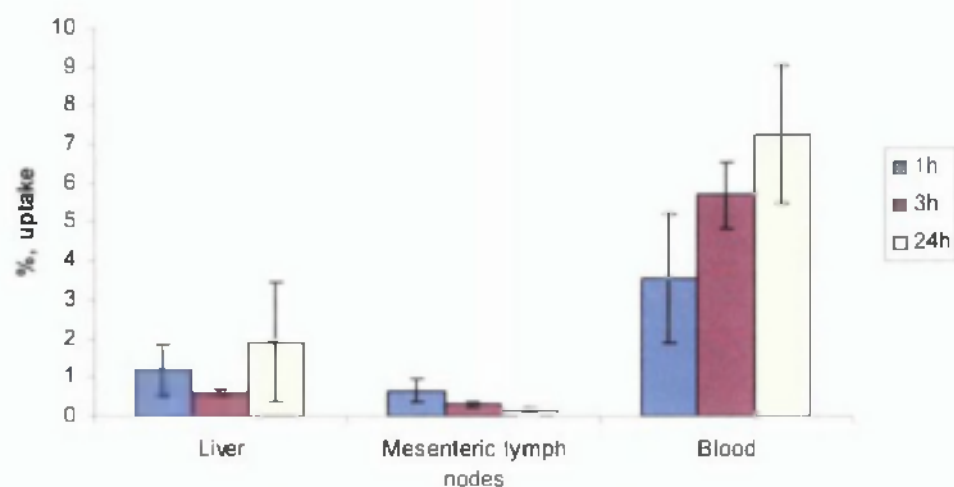


Figure 5.9. Histogram showing the presence of orally administered dendrimer-derived nanoparticles (50 mg/kg) in the liver, mesenteric lymph nodes and blood illustrating the true extent of absorption. Maximum level of dendrimer-derived nanoparticles were found in blood and liver 24 h after administration.

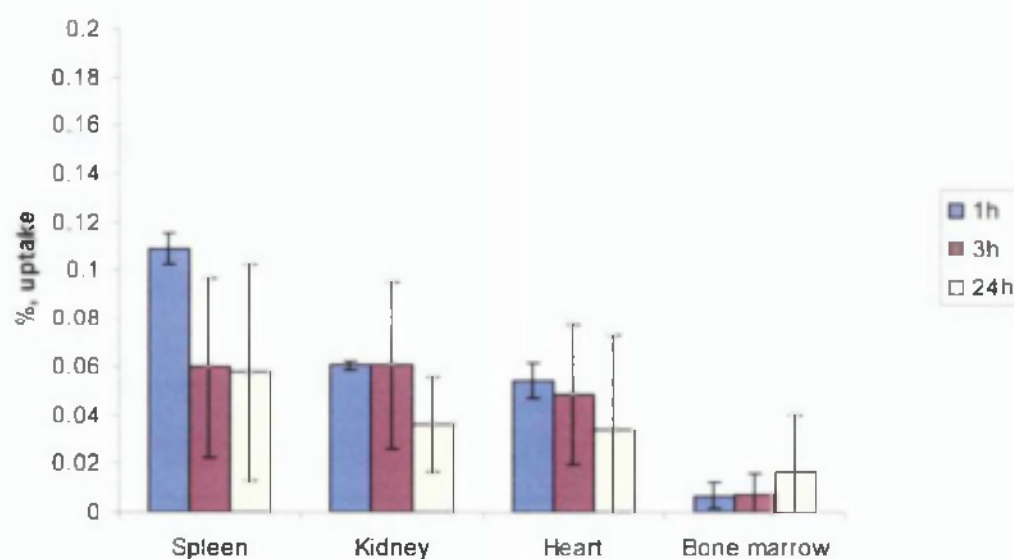


Figure 5.10. Histogram showing the presence of orally administered dendrimer-derived nanoparticles (50 mg/kg) in the spleen, kidneys, heart and bone marrow. The level of uptake in these organs were minimal suggesting a slow clearance of the dendrimer-derived nanoparticles from the blood stream. Maximum level of dendrimer-derived nanoparticles in these organs were found in spleen 1 h after administration.

Size and shape are important parameters which influence the organ distribution of particles. The uptake of particles by for example the liver and spleen has been shown to increase with increasing particle size which was as an effect of faster clearance of larger particles (Davis 1981). The same trend was observed whether they were liposomes (Gregoriadis *et al.* 1977, Senior *et al.* 1985) or fat emulsion droplets (Karino *et al.* 1987). Dendrimer-derived nanoparticles mainly accumulated in the liver, mesenteric lymph nodes and the blood (Figure 5.9). The amount recovered in the liver after 24 h (1.9 ± 1.5 %) was higher than after 1 and 3 h (1.2 ± 0.6 % and 0.6 ± 0.1 % respectively) which might be as a direct effect of slow clearance of small particles. This was further emphasized by the amount of dendrimer recovered in the blood after 1 (~4 %), 3 (~6 %) and 24 h (~7 %), with a significant amount still present in the blood after 24h. This underlines the fact that in order to reduce reticuloendothelial system clearance, small particles are preferred.

Zilversmit *et al.* (1952) studied the rate of size dependent clearance of colloidal gold particles (10 to 40nm) and showed that there was little difference in total uptake of the liver and spleen, indicating that the size mainly influences the kinetics rather than the total uptake. Furthermore colloidal carriers are able to exit the blood capillary system via the sinusoidal fenestrations of the liver and bone marrow, given that they are smaller than the size of the fenestrations (150nm) (Gregoriadis *et al.* 1986). The fact that small amounts of dendrimer-derived nanoparticles were present in the liver, spleen and heart could be a result of a size effect (Figure 5.10). Dendrimer-derived nanoparticles were ~23nm in diameter, with flexible surface lipidic chains which could enable the particles to enter and leave various organs quite rapidly. The recovery in the kidneys was minute (0.06 ± 0.03 % maximum recovery after 3 h), most likely indicating a slow clearance of the dendrimer-derived nanoparticles.

Superimposing the maximum uptake obtained in the spleen and liver of 23nm dendrimer-derived nanoparticles onto Figure 5.11 (modified from Florence *et al.* 2000) it can be determined how the data compares with other particle diameters.

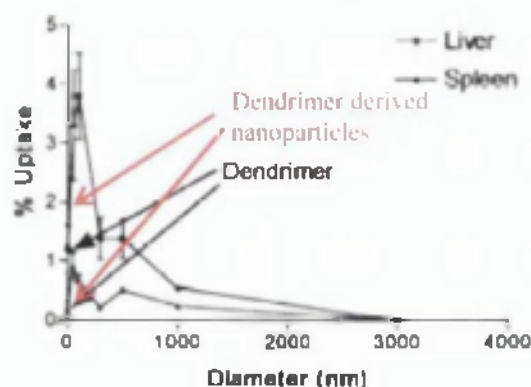


Figure 5.11. Uptake of orally administered dendrimer and polystyrene latex in the liver and spleen as a percentage of administered dose as a function of particles size. The dose of dendrimer was 14 mg/kg (single dose) whereas, polystyrene latex particle were 1.25 mg/kg for 10 days (Taken from Florence *et al* 2000). The results obtained from the presented study are superimposed on to the graph, showing a slightly larger uptake (~2 %) of dendrimer-derived nanoparticles (50 mg/kg, 23 nm) in the liver than dendrimer (2.5 nm). The values fit onto the graph, suggesting that particles between 23 nm and 50 nm are most likely to be taken up by the liver.

Comparing the data, dendrimer-derived nanoparticles, showed no apparent difference in uptake of the spleen while the uptake in the liver was slightly higher than for 4th generation lysine based lipidic dendrimers. The results do suggest that the optimal particle size for particulate uptake of the liver is between 23 and 50nm. The gap seems to be closing, but it is important to remember that uptake and accumulation in the liver is not necessarily favorable for a drug carrier.

The liver (Figure 5.12), kidney (Figure 5.13), spleen (Figure 5.14) and mesenteric lymph nodes (Figure 5.15) from control and dendrimer-treated animals were examined histologically. There was no evidence of dendrimer induced changes in tissues or in the organ weight of the animals (Figure 5.16) at dendrimer concentrations of 50 mg/kg. The results demonstrated that dendrimers taken up in the liver, kidney, spleen and mesenteric lymph nodes did not show any toxicity.

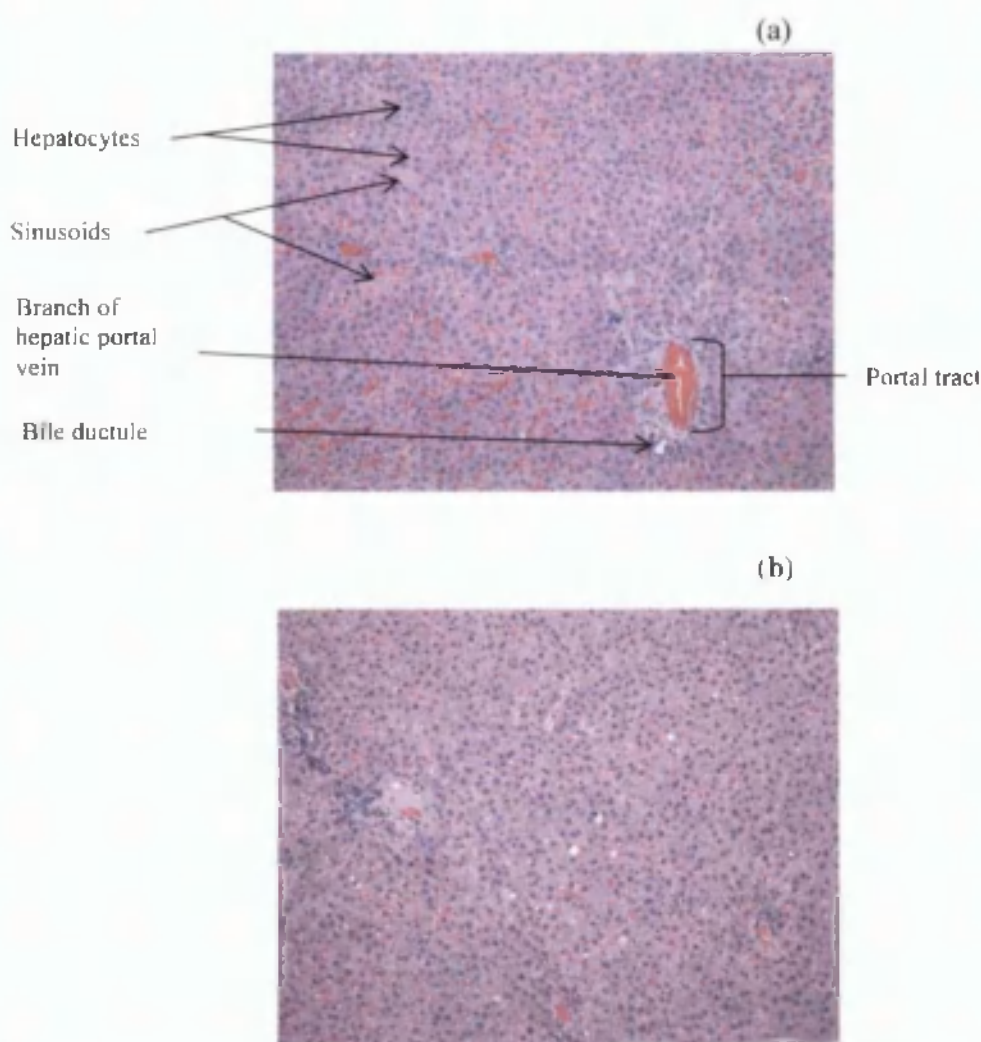


Figure 5.12. Images of sections (3-4 μm) of the rat liver, fixed in 10.5 % Phosphate buffered formalin and H & E stained (X 100). (a) represents dendrimer treated and (b) control. No differences were observed between control and treated animals.

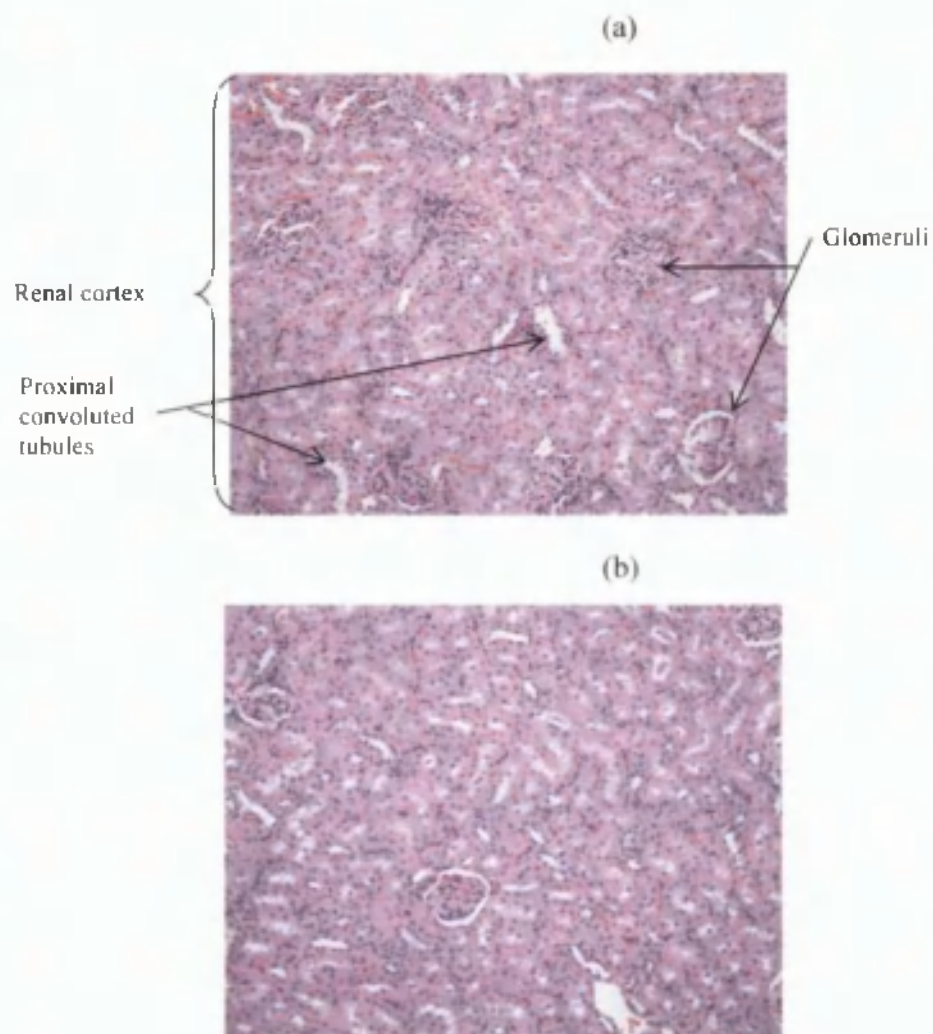


Figure 5.13. Images of sections (3-4 μm) of rat kidney, fixed in 10.5 % Phosphate buffered formalin and H &E stained (X 100). (a) represents dendrimer treated and (b) control. No differences were observed between control and treated animals.

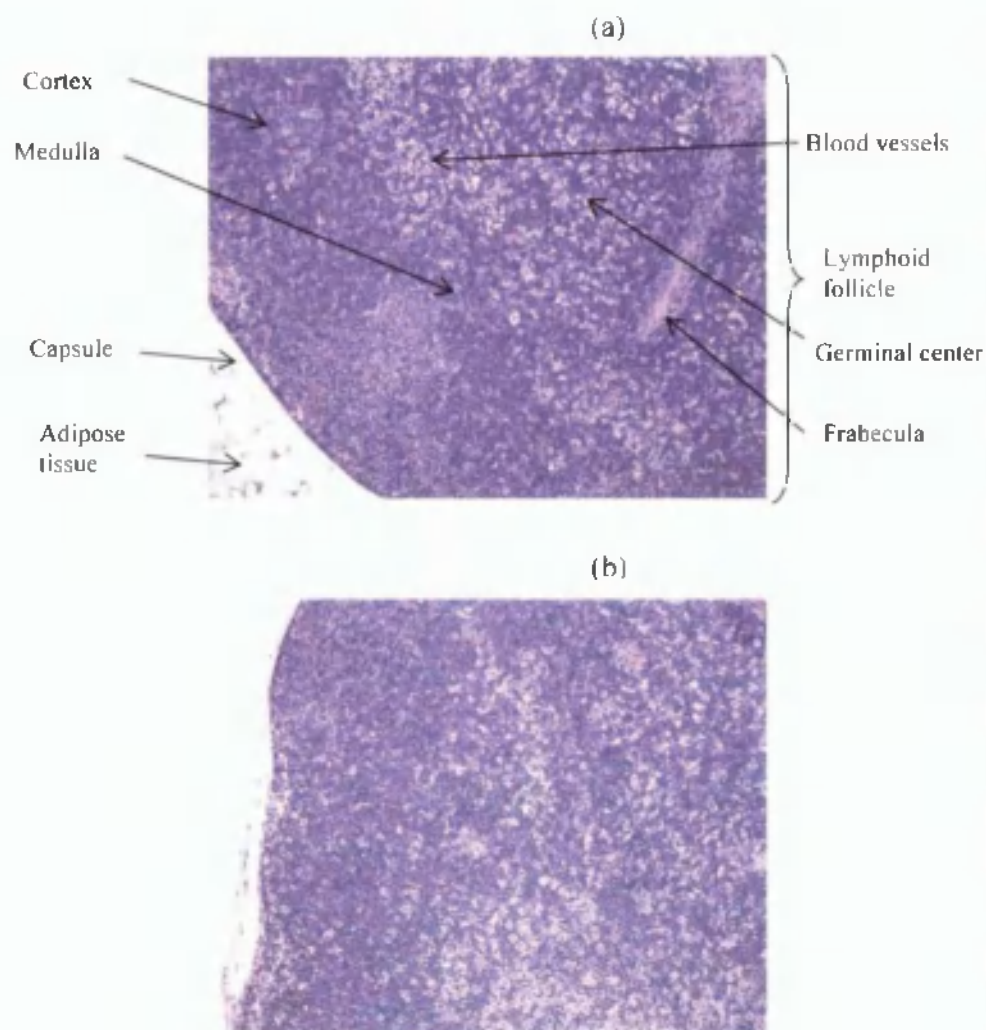


Figure 5.14. Images of sections (3-4 μm) of rat mesenteric lymph nodes, fixed in 10.5 % Phosphate buffered formalin and H & E stained (X 100). (a) represents dendrimer treated and (b) control. No differences were observed between control and treated animals.

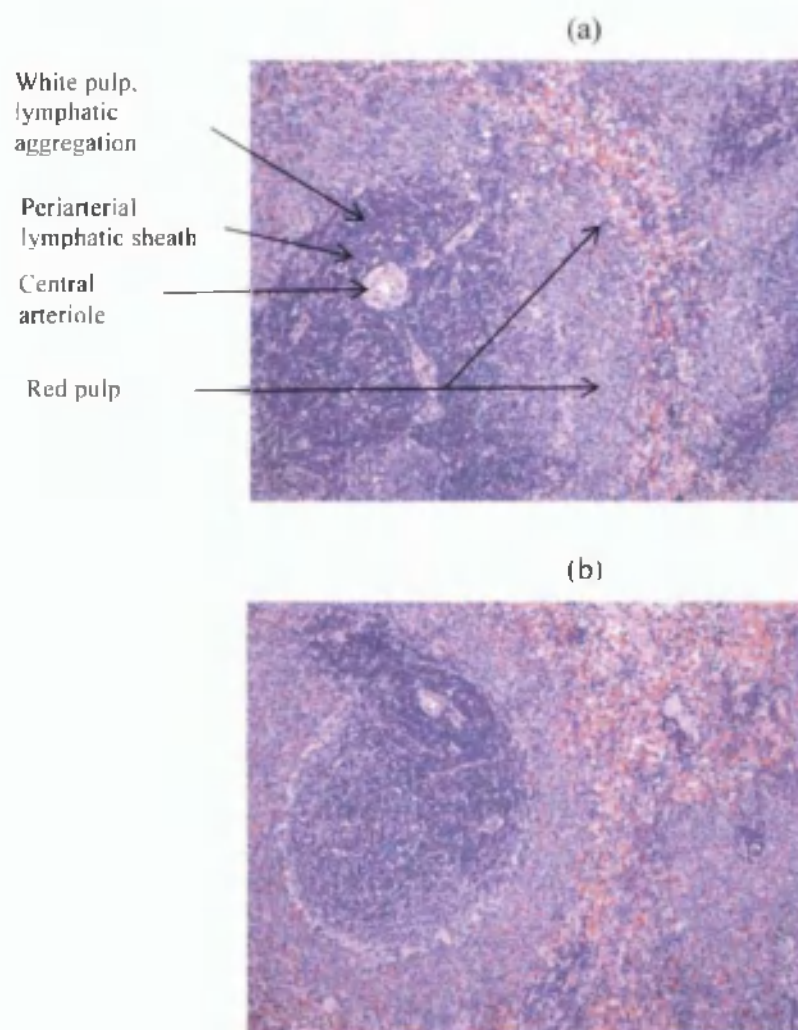


Figure 5.15. Images of sections (3-4 μm) of rat spleen, fixed in 10.5 % Phosphate buffered formalin and H &E stained (X 100). (a) represents dendrimer treated and (b) control. No differences were observed between control and treated animals.

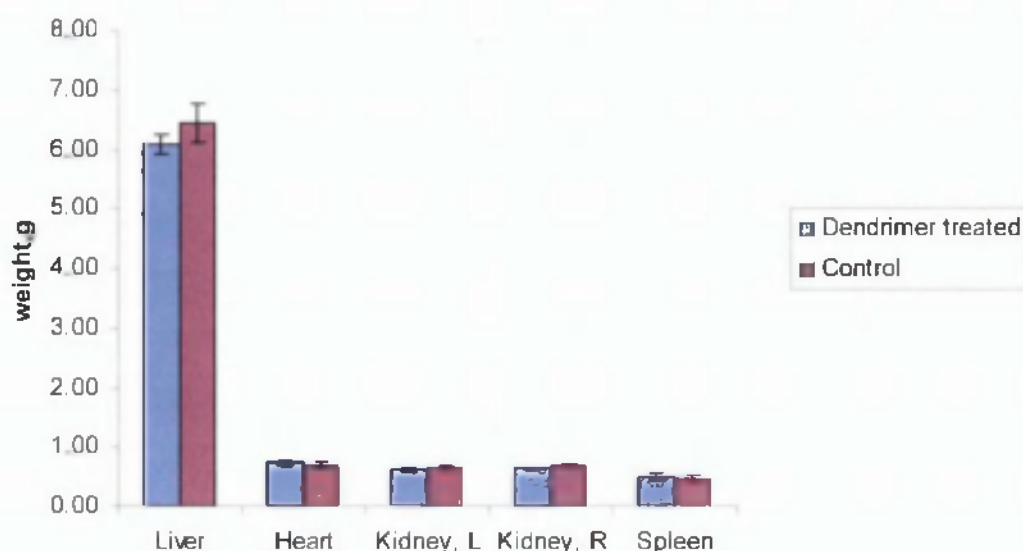


Figure 5.16. Histogram of the weight of the major organs in the abdominal and thoracic cavities after 32 h. Female Sprague-Dawley rats (n=5, control n =3) were dosed with 50 mg/ml dendrimer-derived nanoparticles. No difference in the weight of control animals and dendrimer treated animals was observed and therefore it was concluded that no toxic effect was apparent of the dendrimer-derived nanoparticles on these organs

The total amount of dendrimer-derived nanoparticles recovered in the intestinal organs and the visceral organs (spleen, heart, kidney, mesenteric lymph nodes, blood and bone marrow) at various time points were calculated (Figure 5.17).

As the dendrimer-derived nanoparticles flowed through the intestinal organs there was a steady increase the amount recovered in the visceral organs. This was as explained mainly due to the slow clearance of the dendrimer-derived nanoparticles from the blood. The maximum amount taken up by the visceral organs (at 1, 3 or 24 h after administration) was ~9 %, a significant amount and slightly higher than for the 4th generation lysine based lipidic dendrimer (5 %) (Florence *et al.* 2000) and the 7 % obtained by Jani *et al.* (1989) using 50nm polystyrene nanoparticles. The results suggest that 23nm dendrimer-derived nanoparticles with flexible surface lipidic

chains are translocated more effectively through the PP. For determining the optimal size the gap seems to be closing in, but it is important to remember that size is one of several factors affecting uptake. Charge, surface modification, flexibility etc. are important factors which need to be also studied in detail. The findings here are typical of other studies conducted in our laboratories (and others) over the years.

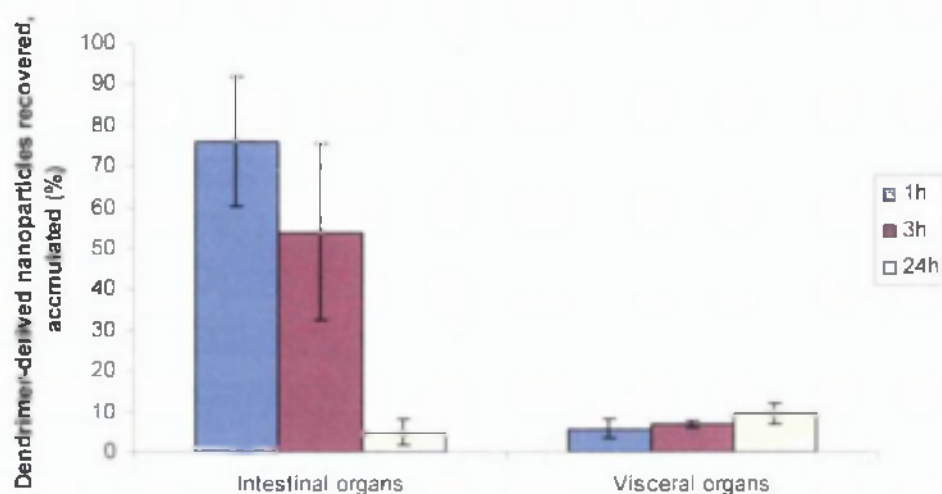


Figure 5.17. Histogram of the total amount recovered in the gastro-intestinal organs (stomach, small intestine, Peyer's patches and large intestine) and visceral organs (liver, spleen, kidney, mesenteric lymph nodes, heart and blood) at various time points. Female Sprague-Dawley rats (n =3) were dosed with 50 mg/ml dendrimer-derived nanoparticles. Most of the dendrimer-derived nanoparticles were recovered in the intestinal organs after 1 h and in the visceral organs after 24 h. the results showed a the movement of particles from the intestinal organs to the visceral organs.

5.4 Conclusion

Nanoparticles, derived from the 5th generation lysine based lipidic dendrimers with a diameter of 25nm, showed evidence of targeting to the PP. A comparatively larger amount of dendrimer-derived nanoparticles were present around the Peyer's patches, compared with the rest of the small intestine. The transit time of the nanoparticles from the stomach through to the large intestine was quite rapid, over 1 h to 3 h. Their recovery from the Peyer's patches and the surrounding area decreased after 3 h.

Given that the recovery of dendrimer-derived nanoparticles from the small intestine was constant over this time period, and that permeability through mucus has been shown to rapid, 2 min for 14nm polystyrene particles (Szantkuti 1997), it is likely that uptake of 23nm particles is rapid, hence a decrease in the recovery from the Peyer's patches is observed.

In the visceral organs, the dendrimer-derived nanoparticles mainly accumulated in the blood with traces found in the liver and spleen. The highest concentration in blood was found after 24 h, which was mainly due to the fact that small particles have slow clearance (Davis 1981). The translocation of dendrimer-derived nanoparticles (23nm) through to the systemic circulation was shown to be higher than for the 4th generation lysine based lipidic dendrimer (2.5nm) (Sakthivel *et al.* 1998), suggesting that there is a optimal size for uptake and translocation of nanoparticles through the PP. The size, hydrophobicity and flexibility of the particles increased uptake and reduced clearance with no toxic effect observed in any organs. The results were very encouraging and even though many problems are yet to be faced, they have laid a foundation for e.g. drug, gene or vaccine delivery using dendrimer-derived nanoparticles.

Conclusion

6.1 Conclusion

The main aim of the work described in this thesis, was to synthesise a series of lipidic dendrimers, subsequently converted into nanoparticles, and to assess this carrier system in terms of oral delivery. The size and packing of the dendrimer-derived nanoparticles combined with their hydrophobicity and flexible surface nature was expected to increase stability in biological fluids, reduce toxicity compared to cationic dendrimers and improve the translocation of these particles through the Peyer's patches of the GALT.

The translocation of polystyrene nanoparticles through the PP of the GALT has been much debated, even though evidence of particulate uptake has been shown for several decades. The extent of uptake however, has not been great. Several factors, such as particle size, hydrophobicity, attachment of bacterial and plant ligands has

been utilised in order to enhance uptake of particles. A biodegradable carrier, translocated in adequate amount has still not been found. In the search for a carrier which is translocated in sufficient amount, particle diameter has been a major area of interest. In order to further investigate the role of particle diameter on translocation, dendrimers were synthesised and constructed into nanoparticles

A series of lipidic lysine based dendrimers were synthesised using Fmoc SPPS. Mass spectrometry confirmed the molecular weight of the dendrimers. With a growing dendrimer generation from 5th to 6th, the coupling efficiency, to produce the dendrimers, decreased. Dendrimers were ellipsoidal in shape for both 5th and 6th generation dendrimers, with their branched structure providing an overall dense structural arrangement. The dendrimers synthesised, were hydrophobic and practically insoluble in aqueous medium. Consequently, dendrimers were formulated into nanoparticles, with diameters ranging from 5 to 50 nm.

The aqueous suspension of nanoparticles was formulated towards the use in oral delivery. The formulation was based on a DCM/water emulsion, in which dendrimers form nanoparticles. The aggregation of dendrimers to form nanoparticles, was mainly governed by their hydrophobic surface nature, with their surface potential contributing to the stability of the particles in suspension. The ultimate particle diameter of the nanoparticles was primarily governed by dendrimer generation. Surface modification of the dendrimers, with lipidic chains C₄ to C₁₂, resulted in the following ranking regarding diameter, C₁₀ > C₁₂ ≈ C₄. Dendrimer (II) with C₁₀ surface lipidic chains formed larger nanoparticles in the series. The larger nanoparticles diameter was suggested to be because of a “looser” packing. Studying the monolayer of the dendrimers at the air/water interface, showed a more expanded

monolayer for dendrimers with C₁₀ surface lipidic chains. This was evident for both the 5th and the 6th generation dendrimers. The other dendrimers in each generation formed more condensed monolayers at the air/water interface and hence the packing into nanoparticles resulted in a smaller particle diameter. Changing the surface nature of the dendrimers, by varying the length of the surface lipidic chains, showed a small and variable effect on particle diameter. The packing and hence the ultimate particle diameter, seemed to be a result of flexibility and hydrophobicity of the surface lipidic chains.

Growing the dendrimer generation from 5th to 6th resulted in the formation of larger particles. The effect was mainly due to the larger diameter of the primary dendrimer. Dendrimers studied at molecular level showed a more expanded monolayer at the air/water interface for 6th generation dendrimers. A more expanded monolayer was likely to suggest that when dendrimers were formulated into nanoparticles they would form larger particles. The experiments underline the importance of studying dendrimers at molecular level in order to understand the parameters influencing the aggregation of dendrimers into nanoparticles.

The diameter of dendrimer-derived nanoparticles could be controlled, by changing the dendrimer concentration. Increasing the dendrimer concentration, decreased the diameter of dendrimer-derived nanoparticles for dendrimers with carbon chain lengths C₁₀ and C₁₂, while a shorter carbon chain length, C₄, resulted in an increased particle diameter. Calculations suggested that the branched dendrimer structure, with lipidic chains extended from their surface, created spaces or voids. The voids combined with hydrophobicity and flexibility of carbon chains provided the opportunities for dendrimer packing. The construction of nanoparticles was mainly

governed by surface flexibility, hydrophobicity and concentration of the dendrimers. Short carbon chain lengths on the surface of the dendrimers, provided a more ridged molecule, and therefore assembled together differently than dendrimers with longer lipidic chains. The resulting effect, for the shortest carbon chain length (C₄), was an increase in particle diameter. Growing the surface carbon chain length formed compact aggregates at a lower dendrimer concentration due to increased hydrophobic interaction.

The diameter and stability of dendrimer aggregates, was dependent on dendrimer generation and concentration. The packing of the dendrimers was mainly through the hydrophobic surface lipidic chains, predominantly forming smaller aggregates of dendrimers. Larger aggregates were also observed in the formulations making up less than 10 % for the 6th generation dendrimers, and less than 5 % for the 5th generation dendrimer. The main issue was the control of the formation of larger aggregates. The potential of dendrimer-derived nanoparticles in drug, vaccine and gene delivery is still to be studied and whether the relatively few larger aggregates obscures results, needs to be assessed for each individual case. However, in the assessment of dendrimer-derived nanoparticles with entrapped drug, the larger particles are primarily more significant, as drug loading probably is likely to be higher in larger particles. Assessing the biodistribution of self-assembled dendrimer-derived nanoparticles after oral administration, larger particles are insignificant, as larger sizes are not taken up by the intestinal epithelium to the same extent.

The toxicity of dendrimer-derived nanoparticles was assessed prior to *in vivo* studies, and was determined by the length of lipidic chains and generation of dendrimers used. Dendrimer-derived nanoparticles from dendrimers with C₁₂ surface lipidic

chains attached showed a comparatively higher cytotoxicity in Caco-2 cells and haemolysis in red blood cells, than dendrimer nanoparticles derived from dendrimers with C₄ and C₁₀ surface lipidic chains. The longer lipidic chain seemed to interact strongly with the cell membranes and hence cause haemolysis or cytotoxicity.

Cytotoxicity in one cell line *in vitro* is not indicative of toxicity *in vivo*. The variation in properties of cells will impart different levels of resistance towards drugs. Furthermore, there is a significant difference in the scale between the area normally utilised *in vitro*, and the total contact area of particles, especially when studying uptake through the intestine.

The dendrimer-derived nanoparticles were stable against degradation in biological fluids. The radioactive label on the dendrimer, did not detach itself when it came into contact with purified intestinal and stomach fluid. This was very important, as it then could be assumed that the dendrimer were intact prior to uptake and translocation. Intestinal and stomach fluid caused aggregation of the system, and was the likely contribution to the protection against degradation, but was also likely to negatively affect the absorption of these systems *in vivo*.

Nanoparticles of 25nm in diameter were derived from the 5th generation lysine based lipidic dendrimers. After oral gavage, a comparative larger amount of dendrimer-derived nanoparticles present around the Peyer's patches, than the rest of the small intestine. The movement of particles through the intestinal organs was quite rapid 1 h to 3 h. During this time-interval, the recovery of dendrimer-derived nanoparticles from the Peyer's patches and the surrounding area decreased, while the recovery of dendrimer-derived nanoparticles from the small intestine was constant. The uptake

through the Peyer's patches seems more prone, especially considering the rapid permeability through the mucus, 2 min for 14nm carboxylated polystyrene particles (Szantkuti 1997). Dendrimer-derived nanoparticles (23nm) would be expected to permeate the epithelial of the Peyer's patches in a similar rapid fashion, hence the decrease in the recovery from the Peyer's patches was argued as better absorption. Saturation of uptake process is also likely, however, given that the amount of dendrimer-derived nanoparticles recovered from the blood increased over the first 3 h, it is likely that the decrease in dendrimer-derived nanoparticles recovered around the Peyer's patches is due to fast absorption.

The major organs of the abdominal and thoracic cavities such as liver, spleen, kidney, heart, mesenteric lymphnodes, bone marrow and blood gives a true indication of the extent of translocation of particles. The dendrimer-derived nanoparticles mainly accumulated in the blood with traces found in the liver and spleen. The dendrimer recovered from the blood increased over 24 h, most likely due to slow clearance of small particles (Davis 1981). The amount of dendrimer-derived nanoparticles (23nm) translocated and recovered in the blood was shown to be higher than for the 4th generation lysine based lipidic dendrimer (2.5nm) (Sakthivel *et al.* 1998), suggestive of an optimal size for uptake and translocation of nanoparticles through the Peyer's patches of the gut associated lymphoid tissue. The effect of particle diameter, hydrophobicity and surface flexibility of dendrimer-derived nanoparticles contributing to increased uptake and reduced clearance with no toxic effect observed in any of the organs studied. The results were positive but many problems are yet to be faced. The realisation of the full potential of dendrimers as carriers will only be achieved through further studies using their drug, gene or vaccine carrying possibilities.

Appendix I

Dendrimer I

Yield 23 %

MS: m/z (%) $C_{316}H_{570}N_{64}O_{64}$ (6290.37): 6313 $[M+Na]^+$ (50), 6116 $[M-3 \times 71.09 (C_4H_7O)+Na]^+$ (63), 5909 $[M-5 \times 71.09+Na]^+$ (75).

1HMR ((D₆) DMSO): 7.83 (d, CONHCH), 7.62 (d, CONHCH), 7.37 (t, CONHCH₂), 7.30 (t, CONHCH₂), 1.49 (m, COCH₂), 1.23 (m, (CH₂)₇), 0.82 (t, CH₃)

Dendrimer II

Yield 26 %

MS: m/z (%) $C_{508}H_{957}N_{64}O_{64}$ (8975.63): 8977 $[M+2H]^+$ (40), 8371 $[M-4 \times 155(C_{10}H_{19}O)+Na]^+$ (61), 8229 $[M-5 \times 155(C_{10}H_{19}O)+Na]^+$ (68).

1HMR ((D₆) DMSO): 7.92 (d, CONHCH), 7.66 (d, CONHCH), 7.48 (t, CONHCH₂), 7.35 (t, CONHCH₂), 1.41 (m, COCH₂), 1.17 (m, (CH₂)₇), 0.79 (t, CH₃).

Dendrimer III

Yield 32 %

MS: m/z (%) $C_{572}H_{1082}N_{64}O_{64}$ (9881.2): 9680 $[M-1 \times 183(C_{120}H_{23}O)]^+$ (30), 9494 $[M-2 \times 183(C_{120}H_{23}O)]^+$ (35), 9154 $[M-4 \times 183(C_{120}H_{23}O)]^+$ (40).

1HMR ((D₆) DMSO): 7.74 (d, CONHCH), 7.48 (d, CONHCH), 7.30 (t, CONHCH₂), 7.17 (t, CONHCH₂), 1.23 (m, COCH₂), 1.01 (m, (CH₂)₇), 0.61 (t, CH₃)

Dendrimer IV

Yield 12 %

MS: m/z (%) $C_{636}H_{1144}N_{128}O_{128}$ (12590.8): 12612 $[M+Na]^+$ (25), 11965 $[M-9 \times 71.09 (C_4H_7O) + Na]^+$ (20), 11660 $[M-13 \times 71.09]^+$ (23), 10826 $[M-25 \times 71.09 + Na]^+$ (39).

1HMR ((D_6) DMSO): 7.99 (d, CONHCH), 7.73 (d, CONHCH), 7.54 (t, CONHCH₂), 7.41 (t, CONHCH₂), 1.50 (m, COCH₂), 1.25 (m, (CH₂)₇), 0.84 (t, CH₃)

Dendrimer V

Yield 16 %

MS: m/z (%) $C_{1020}H_{1912}N_{128}O_{128}$ (18019.1): 18060 $[M+ 2Na]^+$ (32) 13807 $[M-27 \times 155(C_{10}H_{19}O)]^+$ (91).

1HMR ((D_6) DMSO): 7.92 (d, CONHCH), 7.65 (d, CONHCH), 7.47 (t, CONHCH₂), 7.34 (t, CONHCH₂), 1.40 (m, COCH₂), 1.16 (m, (CH₂)₇), 0.78 (t, CH₃)

Dendrimer VI

Yield 14 %

MS: m/z (%) $C_{1148}H_{2168}N_{128}O_{128}$ (19814.5): 19390 (52), 18982 $[M-4 \times 183(C_{120}H_{23}O) + 4Na]^+$ (48), 17983 $[M-10 \times 183(C_{120}H_{23}O)]^+$ (58), 15408 $[M-24 \times 183(C_{120}H_{23}O)]^+$ (72).

1HMR ((D_6) DMSO): 7.98 (d, CONHCH), 7.73 (d, CONHCH), 7.55 (t, CONHCH₂), 7.42 (t, CONHCH₂), 1.48 (m, COCH₂), 1.24 (m, (CH₂)₇), 0.86 (t, CH₃)

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