STUDIES OF DIFFUSION IN CELLS USING A SELF-FLUORESCENT DENDRIMER

A thesis presented by

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This thesis describes research conducted in the School of Pharmacy, University of London between November 2003 and July 2007 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.

July 2007

_________________________  ________________________
Signature                  Date
“I have made this thesis longer only because I have not had time to make it shorter.” Will apologies to Blaise Pascal, Letters Provinciales, 1657
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ABSTRACT

Dendrimer synthesis and potential applications of the lysine-based dendrimers as gene carrier systems and nano-fluorescent probes have been explored. An intrinsically fluorescent polylysine dendrimer (DM1), (Gly)(Lys)_{63}(NH_{2})_{64}, was synthesized by both Fmoc- (30% overall yield) and Boc-SPPS (64% overall yield) by divergent method and characterized by NMR, MS and RP-HPLC. Boc-SPPS is a recommended method for synthesizing this compound which provided higher yield and purer product.

Diffusibility of the DM1 in various types of media and in cells has revealed the difference between \textit{in vitro} and \textit{in vivo} transport. In concentrated and more complex media (20-60%(v/v) glycerol solutions, HPMC and actin gels) when beyond the limits of PCS, the FRAP technique allowed the diffusion coefficients (D) of DM1 to be accessed. For DNA delivery, DM1, DM2 ((C_{18})(Lys)_{7}(NH_{2})_{8}) and DM3 ((C_{18})(Lys)_{7}(NH_{2})_{8}(RGD)_{3}) were capable of condensing DNA. The DNA was better condensed by DM2 and DM3 possibly due to the synergistic effects between the electrostatic and hydrophobic interactions. At charge ratios of 20:1 (DM1) and 10:1 (DM2 and DM3) (+/-) a 4h incubation period produced optimal transfection. Confocal microscopy and luciferase assay indicated transfection efficiencies of the dendrimers in descending order: DM3>DM2>LPP(Lipofectamine-Plus\textsuperscript{TM})>DM1>pDNA. As a nano-fluorescent probe (DM1), the method for studying the dynamic uptake of this fluorescent probe has been established and allowed the D values to be calculated. Values were found in the range from 5.99\times10^{-11} \text{cm}^2 \text{s}^{-1} (in SK/MES-1 cells) to 9.82\times10^{-11} \text{cm}^2 \text{s}^{-1} (in Caco-2 cells) for the dendrimer and 2.36\times10^{-11} \text{cm}^2 \text{s}^{-1} (in Caco-2 cells) for the dendriplexes. The difference implied variation in the intracellular architecture of the cell types and the effect of particle size. Using the DM1 with other fluorescent dyes the excitation/emission spectra of the dyes and the concentration used of the DM1 must be considered to avoid the contribution of the DM1 in emission spectrum of the dyes. The uptake of both dendrimer and dendriplexes entailed endocytosis which was reduced by the effect of medium flow. The DM1 dendrimer clearly interacted with actin filaments via the electrostatic interaction as confirmed by the TEM, SEM and SDS-PAGE results. Actin formed reversible insoluble complexes with DM1 indicating none of chemical bonding within the complexes reinforced by results from Western blots. DM1 exhibited biphasic effects on the actin polymerisation process depending on its concentration. It displayed inhibitor and activator behaviour, respectively, at low and high concentration.

It was concluded that DM1, DM2 and DM3 serve as potential tools for gene delivery whereas their unique architectures still allow them to be engineered for indefinite applications in the future.
ABBREVIATIONS

ALN Amino latex nanospheres
AT Actin monomer (G-actin)
Boc tert-Butoxycarbonyl
CLN Carboxyl latex nanospheres
CSLM Confocal scanning laser microscopy
DIW Double distilled deionised water
DCM Dichloromethane
DMF Dimethylformamide
D Diffusion coefficient
Dg Diffusion coefficient in gel
dc cyto Diffusion coefficient in cell cytoplasm
DIEA N,N-diisopropylethylamine
DM1 (Gly)(Lys)6(NH2)64 dendrimer
DM2 (C18)(Lys)7(NH2)8 dendron
DM3 (C18)(Lys)7(NH2)7(RGD) dendron
DMEM Dulbecco’s modified Eagle medium
DMSO Dimethylsulfoxide
FBS Foetal bovine serum
Fmoc 9-Fluorenylmethoxycarbonyl
FRAP Fluorescent recovery after photobleaching
HBSS Hank’s balanced salt solution
HBTU O-benzotriazolyl-N,N,N’,N’-tetramethyluronium hexafluorophosphate
HF Hydrogen fluoride
HPMC Hydroxypropyl methyl cellulose
HOBT 1-hydroxybenztriazole
Hoechst 33352 Trihydrochloride trihydrate
IC50 Fifty percent inhibition concentrations
LDNA Fluorescent labelled DNA
LinLys Linear polylysine
LPP Lipofectamine-Plus™ transfecting agent
MALDI-TOF Matrix-assisted laser desorption ionization-time of fly
MBHA 4-Methylbenzhydramine
MEM MEM non essential amino acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NA</td>
<td>Numerical aperture</td>
</tr>
<tr>
<td>NLP</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methylpyrrolidone</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Poly(amidoamine)dendrimers</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer solution</td>
</tr>
<tr>
<td>PCS</td>
<td>Photon correlation spectroscopy</td>
</tr>
<tr>
<td>pDNA</td>
<td>Plasmid DNA</td>
</tr>
<tr>
<td>PLL</td>
<td>Poly-l-lysine</td>
</tr>
<tr>
<td>PPI</td>
<td>Polypropyleneimine</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse phase high performance liquid chromatography</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative luciferase units</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid phase peptide synthesis</td>
</tr>
<tr>
<td>SPS</td>
<td>Solution peptide synthesis</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>WGA</td>
<td>Wheat germ agglutinin- Alexa Fluor® 595 conjugate</td>
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Chapter I

INTRODUCTION

This thesis focuses on a dendrimer-based nanocarrier system in various aspects: i) dendrimer synthesis and characterisation; ii) diffusion behaviour of dendrimers in bulk media, polymer solutions and gels and in natural cellular protein networks and the cytosol; iii) formulation and transfection abilities of dendriplexes; iv) dynamic uptake of the dendrimer and dendriplexes by cells; and v) the ability of the dendrimer to interact with cellular organelles (e.g. actin cytoskeleton). This introduction provides general background knowledge and the circumstances of the work.

A fascinating aspect now renowned as a fundamental concept of “nanotechnology” was first introduced in 1959 by the Nobel-prize winning physicist Richard Feynman (Feynman 1960). In his famous speech “there is plenty of room at the bottom” he conceived a time when visualisation, manipulation, data storage, and manufacture would be feasible on the atomic scale. Matters reduced into a proper small-scale, can readily present very unusual properties compared to what they exhibit on a macro-scale, enabling new unique applications and possibilities. For example, the stable form of aluminum turns combustible; an insulator (silicon) transforms into a conductor; the opaque (emulsion) becomes transparent. These concepts have rapidly invaded a wide range of subjects and elicited the birth of the technological innovations in 20\(^{th}\) century such as the laptop computer, mobile phone, scanning tunneling microscopy (STM), atomic force microscope (AFM), and digital versatile disc (DVD) which all dramatically improve the quality of human life. Medicine is a fertile area for this invasion.
1.1 Nanomedicine and the future medicine

The term “nanomedicine” (drug more complex than conventional ones) is simply nanotechnology applied to the medical area, encompassing areas of nanocarrier systems, ranging between 1-100 nm, for drug/gene delivery, targeting, diagnosis, vaccinology and feasible future applications of molecular nanotechnology. It is aimed to be more rapid, accurate, selective and effective in diagnosis, monitoring and treatment without side effects, and allow these process to be evaluated noninvasively (Freitas 2005b; Caruthers et al. 2007). At the nanoscale, the fundamental properties and bioactivity of drug can be altered, permitting a control over various characteristics of drugs such as solubility, blood pool retention times, controlled release effect, environmentally triggered controlled release or highly specific site-targeted delivery.

1.1.1 Why smaller?

The smaller the particles, the greater the surface area to volume ratios, and the higher the chemical reactivities and some biological activities. A particularly small size of nanoparticles also implies that they can be taken up more readily by the human body than larger sizes. Nanomaterials are capable of crossing biological membranes and entering cells, tissues and organs whereas larger-sized particles typically cannot. On the contrary, size also displays as a key feature in determining the potential toxicity of particles. Some nanoparticulates have been proved to be toxic to human tissue and cell cultures, rendering an increase in oxidative stress, inflammatory cytokine production and cell death (Oberdörster et al. 2005). Practical implementation of an effective nanocarrier system requires a balance between its therapeutic and toxic effect.

Figure 1.1 summarises some of the known influences of the particle size of carriers, ranging from drug loading and release to attach to endothelial surfaces, but the effects range wider (Florence & Ruenraroengsak in preparation). It is generally clear from basic physical principles what is the role of particle size (or mean particle size) in each of these processes (Florence 2004) but there are subtle effects such as size effects in cytoplasmic transport, where the barrier to delivery is the molecularly crowded interior of the cells. The physical and biological factors affecting nanoparticle uptake
before and after transcytosis and translocation have been well documented (Florence 2004; Florence 1997; Florence & Hussain 2001). The volumetric capacity of any carrier system which encapsulates drug is usually of key importance (Ricci et al. 2004; Zhang & Feng 2006): a balance must be struck between the optimal size to achieve transport to desired sites, the avoidance of loss through extravasation and the capacity of the system to hold and retain active molecules. Degradation is often a surface phenomenon, so large surface areas can lead to degradation at a less than optimal rate (Mladenovska et al. 2002). It is straightforward to produce a rational scheme for the sequential processes involved but so far no predictive theory has emerged to allow prediction. The first stage is to examine the processes, obvious and otherwise, which determine delivery. There might be an optimal radius of particles bearing surface ligands to interact with cell surface receptors (Kotani et al. 1962; Waterman & Sutton 2003) and this hints at a new era of molecular pharmaceutics. Nanoparticles of optimal size coated with surface ligands at the appropriate spacing binding ultimately to receptors (Rensen et al. 2001; Fischer et al. 2002) an approach possible as the dimensions of receptors are increasingly known to less than 1 nm (Florence 2004).

1.1.2 Current and prospective carrier systems for drug and gene delivery

Medicine is on the brink of a new epoch - DNA (~2.4 nm across) has transpired as an active therapeutic molecule in gene therapy, and the traditional tablet dosage form has in some cases been transformed to nanodrops and potent nanoparticles (Figure 1.2-1.3). To achieve high efficiency, the active therapeutic agent within carriers has to be protected from the external environment until reaching the target site and once there produce maximal effects and low side effects when released. An assortment of nanocarrier system has, hitherto, been developed to accomplish these goals as illustrated in Figure 1.2. These include liposomes (10nm-100μm); multilamellar vesicles (MLVs) and small unilamellar vesicles (SUVs); micro and nanoemulsions; micro and nanospheres; nanoparticulate drug carriers covering area of polymeric nanoparticles, solid lipid nanoparticles (SLN) (50-100 nm), nanosuspensions (200-600 nm), nanotubes (1-10nm) and quantum dots (1-10 nm). Currently, the FDA (USA) has recounted that 75% of research studies, 59% of patents and 20-30% of all approved drugs are related to nanomedicine (Editorial 2007; Eaton 2007). The spotlight has been
placed on nanosystems for cancer treatment, diagnosis and gene therapy with liposomes, polymeric nanoparticles, nanoshells, nanotubes, quantum dots, and dendrimers (Figure 1.3A).

![Particle size comparison chart]

**Figure 1.1** A summary of the generally known influences of the particle size of drug carrier systems, here small particle and large particle are particles which have mean diameters < 0.5 μm and ≥ 1 μm, respectively.

Prospectively, more attention will sagaciously have to be placed on hybrid systems and multifunctional vehicles for drug/gene delivery and targeting. Such systems might include nanoparticles encapsulated within liposomes, combining the benefit of the long circulation times of modified liposomes with the advantage of encapsulation in nanoparticles. In gene therapy, a hybrid between viral and non-viral vectors is expected to benefit high gene transfection with minimal toxicity and side effects. Instead of directly combining two systems, in which it may be difficult to control physicochemical properties and biological activity; one carrier system can be engineered to achieve the similar goal as a multifunctional carrier (Figure 1.3B).
requires the quest of potential carriers which can be successfully engineered. Dendrimers are likely to be compatible with this strategy according to their unique properties, as addressed in the next section. In addition, a prototype of “microbivores”-nanorobotic artificial phagocytes - has recently been proposed and this science fiction robot may turn real in ten-year timescale (Figure 1.4) (Freitas 2005a).

Figure 1.2 The diversity of carrier systems whose particle size over a 2,000 fold range may be crucial to their performance, ranging from nanocrystals to tableting drug excipients in comparison to size of biological cells and their active/drug molecules presented here as proteins and DNA. The range of pharmaceutical constructs can be seen as well as the modes of particle size measurement adopted in the different size ranges¹. Nanomedicine is allocated the peach zone. (Mollet & Grubenmann 2003)

¹ Different methods of particle size measurement provide different mean values. It is not always apparent from the literature which size measurements have been made and which mean values are quoted, hence it is sometimes not possible to compare directly experiments. A discussion of the methodology adopted is outside the scope of this thesis.
Figure 1.3 The current nanocarrier systems which have been intensively studied in nanomedicine (A) are smaller than human cells and pathogen organelles, but comparable in size to large biological macromolecules such as enzymes and receptors. A multifunctional nanocarrier system in nanomedicine (B): the system is based on a nanoparticle with an exceptional freedom to engineer its surface with multifunctional ligands offering possibilities of nano-biosensors, molecular labelling and tracking, imaging and diagnostic agents, gene/drug delivery, molecular targeting and other biological tools, which promises to impact drug development, medical diagnostics, and clinical applications (McNeil 2005).

Figure 1.4 Nanomedicine in future: a “microbivorse”-nanorobotic artificial phagocytes patrolling bloodstream searches for pathogens to capture and digest. This nanorobot would be expected to accomplish complete clearance of the most severe septicemic infections within an hour or less, according to Freitas. This process takes much shorter than the normal treatment of antibiotic lasting for weeks or months (Freitas 2005a).
1.2 Description of dendrimers

Dendrimers are three dimensional hyperbranched macromolecules which have now been recognized as the fourth class of polymer- “dendritic polymer” - after the conventional linear (i), cross-linked (ii) and branched (iii) polymers (Tomalia 2005). These hyperbranched macromolecules were first introduced as “cascade molecules” in 1978 by Vögtle’s group (Buhleier et al. 1978). The report explained the concept of the synthetic pathway permitting a repetitive growth of the macromolecule with branching from monoamine or diamines. Later this method was developed further and referred to as “a divergent strategy” for dendrimer synthesis (Tomalia et al. 1985). The name “dendrimer” comes from the Greek words: dendri (tree-like) and meros (part of) was first addressed by Tomalia in 1985 (Tomalia et al. 1985). The term “arborols” was used by Newkome (Newkome et al. 1985; Newkome et al. 1986) to explain dendrimer (“arbor” in Greek means branch and in Latin means tree). Dendrons are partial dendrimers. Further reviews of the historical aspects of the subject can be seen elsewhere (Matthews et al. 1998; Tomalia 2005; Fréchet & Tomalia 2001).

The architecture of the dendrimer can be dissected into three distinct parts: core, branching units and the surface groups as can be seen in Figure 1.5A. Such well defined architecture means that the number of the surface groups and branch units in each generation and the molecular weight of the dendrimer can be theoretically predicted (Figure 1.5B). The central core covalently linked to branching unit toward the surface groups will influence the size, shape and directionality of the dendrimer. Branching units locate within dendrimer interior defining the type and amount of interior cavity (void volume) which may be enclosed by the surface groups of high generation dendrimers. The void volume, which can be extended for guest-host properties of the dendrimer, is restricted by dendrimer family and generation. The surface groups can exhibit either as the template polymerisation within the interior of the dendrimer depending on their functional groups, or as the passive/reactive gates and rigid scaffolds (shell) at the outer surface controlling the entry and exit of guest molecules.
Number of Surface Groups \( Z \) = \( N_c N_b^G \)

Number of Branch Cells \( (BC) \) = \( N_c \left\lfloor \frac{N_b^{G-1}}{N_b - 1} \right\rfloor = \text{Number of bonds per generation} \)

Molecular Weights \( (MW) \) = \( M_c + N_c \left[ M_{RU} \left( \frac{N_b^{G-1}}{N_b - 1} \right) + M_t N_b^G \right] \)

Where \( N_c \) = core multiplicity, \( N_b \) = branch-cell multiplicity and \( G \) = generation of the dendrimer; \( M_c \), \( M_{RU} \) and \( M_t \) = molecular weight of core, the repeat branch units and terminal units respectively (adapted from Tomalia, D.A., 2005).

Figure 1.5  Dendrimer structure (A) and mathematical expression of the dendritic architecture (B). Each dendrimer is composed of three parts: core, branching units and the surface or terminal groups. Theoretically, a core is covalently anchored by root attachment points exhibiting as an ordinary generation 0 dendrimer. Herein the core contains tri-root attachment points. The counting of generation 0 usually refers to the core but in some case it encompasses the core after the first coupling reaction of branch units. A number of branching units and the symmetry of the dendrimer construction are restricted by the valency of the core molecule. Dendrimer synthesis involves a repetitive coupling reaction allowing the addition of branch units to the core generation by generation to achieve the desired generation. A dendron is a partial dendrimer (right). Pictures here demonstrate the 5th generation (G5) dendrimers. The hypothetical number surface groups and branch units of each generation and molecular weight of the dendrimer can be directly calculated (B) when the number of generation, core structure and branch unit structure are known. There is a stage that the growth of the dendrimer can not be continued because the increase in molecular density at the dendrimer surface and steric hindrance of these terminal groups which result in insufficient space to accommodate the additional units. This state is so-called a “dense-packed state” (de Gennes & Hervet 1983) and the surface groups at this state serve as a “rigid scaffold” or “shell” allowing extremely slight access to the dendrimer interior (Hecht & Frechet 2001). The figure is adapted from Dufès et al. (2005) and Tomalia et al (2007).

As the generation is increased the surface groups become more rigid. The growth of the dendrimer is self-limiting and restricted by steric hindrance from the increase in molecular density at the dendrimer surface. The stage that the growth of the dendrimer
can not be continued is the “dense-packed state” (de Gennes & Hervet 1983). The surface groups at this state serve as a “rigid scaffold” or “shell” allowing only slight access to the interior of the dendrimer (Hecht & Frechet 2001). The interior cavity can be used to accommodate the guest molecules. Dendrimers can be synthesised by two strategies: divergent and convergent; the detail of dendrimer synthesis is explained in Chapter II.

1.3 Unique properties of dendrimers

The term “macromolecular isomerism” is used to describe macromolecules constructed in the same proportions of the same monomer compositions, yet in different architectural or configurations, and expected to behave differently (Dvornic & Tomalia 1996; Tomalia et al. 1997). The linear and dendritic polymer synthesized as isomers of each other were employed to study the influence of the architecture on their physical properties namely conformation, crystallinity, solubility, intrinsic viscosity, rheology, diffusion/mobility and electronic conductivity as shown in Table 1.1 (Wooley et al. 1994; Hawker et al. 1997; Hawker et al. 1995; Farrington et al. 1998). The dendritic architectures showed amorphous character and remarkably 40% lower hydrodynamic volume compared to the linear architectures (Wooley et al. 1994).

By comparison with the linear polymer, whose microviscosities increase linearly with molecular weight, the dendrimers display maximum and minimum microviscosities at specific generations (Tomalia et al. 1990; de Brabander-van den Berg & Meijer 1993). This is explained by the changes in dendrimer shape from the flexible oval shape to rigid spherical shape as the dendrimer is grown. The rigid spherical shape is devoid of entanglement whereas this phenomenon is prominently found in linear polymer solutions when their mass is augmented (Hawker et al. 1995; Farrington et al. 1998). Moreover, dendrimers have higher solubilities than their isomers in linear constructs and, fact being confirmed by a number of groups (Fréchet 1994).

Unlike conventional polymers where applications are limited by their high polydispersity, the dendrimers possess a high degree of molecular uniformity reflecting in their low polydispersity elucidated by mass spectrometry, size exclusion chromatography, electrophoresis and transmission electron microscopy (TEM).
Concomitantly, the unimolecular container/scaffolding properties of the dendrimer have been investigated and appreciated in nanomedicine. This container property is governed by types and dendrimer generation as presented in Figure 1.6. The dendrimers at lower generations will exhibit as an overt-planar oval shape present lack of the container property while at the intermediate generations the surface groups serve as a “passive or reactive gates” to control the entrance and departure of the guest molecules exhibiting their “container properties”. At the higher dendrimer generations, the surface groups become more rigid and act as the egg-shell-like structure (shell) which can be used to encapsulate drug molecules (Maciejewski 1982). The feature of high peripheral density with the relatively high free space surrounding the dendrimer core was also proposed as a concept of “dendritic box”. Interactions between host and guest molecules have been elucidated (Jansen & de Brabander-van den Berg 1995a; Jansen & de Brabander-van den Berg 1995b).

**Table 1.1** Influence of polymer architecture on physical properties of polymers

<table>
<thead>
<tr>
<th>Physical properties</th>
<th>Linear Polymer</th>
<th>Dendritic Polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Flexible coil</td>
<td>Dendrons Dendrimers</td>
</tr>
<tr>
<td>1. Conformation</td>
<td>Random coil</td>
<td>Predictable shape changes depending on family and generation (elliptical-spherical)</td>
</tr>
<tr>
<td>2. Crystallinity</td>
<td>Semicrystalline/crystalline materials (high glass temperatures)</td>
<td>Amorphous materials (lower glass temperatures)</td>
</tr>
<tr>
<td>3. Solubility</td>
<td>Lower solubility (decrease with MW)</td>
<td>Higher solubility (increase with MW)</td>
</tr>
<tr>
<td>4. Intrinsic viscosity</td>
<td>Increase with MW</td>
<td>Exhibit maximum and minimum at specific generations (independent of MW)</td>
</tr>
<tr>
<td>5. Rheology</td>
<td>Shear sensitivity (entanglement governs rheological properties)</td>
<td>Newtonian-type, no shear sensitivity, lower viscosity</td>
</tr>
<tr>
<td>6. Diffusion/mobility</td>
<td>Reptation (segmental mobility)</td>
<td>Non-reptation (kinetic flow unit)</td>
</tr>
<tr>
<td>7. Electronic conductivity</td>
<td>Anisotropic</td>
<td>Isotropic</td>
</tr>
</tbody>
</table>
Figure 1.6 Nanocontainer properties of dendrimers and dendrimer growth of PAMAM dendrimers (adopted from Tomalia 2005). The lower dendrimer generations present as flexible open-oval shapes. At intermediate generation the surface groups serve as a “passive or reactive gates” controlling the entrance and departure of the guest molecules whereas the interior cavity can be used to accommodate the guest molecules. As the generation is increased the surface groups become more rigid. At the higher generation the surface groups in this state serve as a “rigid surface scaffolding” or “egg-shell-like structure” allowing extremely low access to the dendrimer interior (Hecht & Frechet 2001). Z represents as surface groups of the dendrimers (Tomalia 2005).

Drug encapsulations inside dendrimer have been recognized in two categories 1) the drug molecule can be covalently bound with dendrimer molecule and 2) physically entrapped within dendrimer interior (Lui & Fréchet 1999; Gillies & Fréchet 2005). The paradigm of using dendritic nanocontainers to increase hydrophobic drug encapsulation has been initiated by synthesis of dendrimers with hydrophobic cores and hydrophilic outer surfaces so called “unimolecular micelle” (Stevelmans et al. 1996). In contrast to conventional micelles, such a dendrimer is devoid of a critical micelle concentration (CMC) offering the encapsulation of the hydrophobic drugs at all dendrimer concentrations (Najlah & D’Emanuele 2006).

The persistent nanoscale geometry of the dendrimers provides an aspect of “artificial proteins” (Tomalia et al. 2003) which is applicable for gene therapy and...
immunodiagnostics and other nanomedical applications. However, a disparity in the similarity between the globular protein and the dendrimer can be found: dendrimers possess a core-shell structure, adaptable surface functionality, tunable interior void spaces (cavities) for guest molecules and they can be synthesised with a broad range of non-amino acid components providing non-immunogenicity (Tomalia et al. 2007).

Dendrimers contain highly terminal surfaces which are concomitant with the amplification of the surface groups upon dendrimer growth. This shows great potential ability for multi-surface engineering to provide a variety of applications: in drug (Gillies & Fréchet 2005; Gupta et al. 2006; Cheng et al. 2007) and gene carriers (Paleos et al. 2007; Dufes et al. 2005), in light-harvesting (Balzani et al. 2003), as antimicrobial agents (Klainert et al. 2006), antineoplastic agents (Tam et al. 2002), to enhance the solubility of drug as dendrimer prodrugs (Najlah et al. 2007), as catalysts (Méry & Astruc 2006), artificial enzymes/proteins (Darbre & Reymond 2006), as image contrast agents for diagnosis (Tomalia et al. 2007; Kobayashi & Brechbiel 2005; Venditto et al. 2005), and for imaging and radio cancer therapy (Mitra et al. 2006).

A legendary example is that the dendrimers exhibit artificial light-harvesting antennae (Balzani et al. 1998; Kawa & Fréchet 1998) which possess architectural similarity to chromophore aggregation occurring in natural photosynthesis (Mcdermott et al. 1995; Kühlbrandt 1995). The broad spectrum of light is absorbed at the dendrimer surface groups and transferred to the core before it is converted into the narrow emission spectrum. The light harvesting ability of the dendrimer is found enhanced as the dendrimer is grown due to amplification of the surface groups (Gilat et al. 1999; Adronov et al. 2000; Klajnert & Bryszewska 2001). Recent developments of this application can be seen elsewhere (Balzani et al. 2003; Balzani et al. 2007). The existence of numerous terminal groups of dendrimers aids multiple interactions and binding affinities of the dendrimers with solvents and other molecules (Mammen et al. 1998) as well as with cell membranes, cell organelles and DNA (Bielinska et al. 1996). A review can be found in Chapter VI of this thesis. In gene therapy, the
interaction between the polyvalent dendrimers with cell membranes may facilitate cell entry of dendriplexes (dendrimer-DNA complexes).

1.4 Gene therapy concept and challenges
Gene therapy involves the treatment of human diseases using genetic materials as active therapeutic agents which are transferred to target cells of patients (Mulligan 1993). The developments in biotechnology, molecular biology and the completion of human genome project (Macilwain 2000) allow the identification of both diseases, particularly, genetic diseases and the gene or genes responsible for the disease. The diseases theoretically can be treated by a replacement of the defective gene with a normal healthy gene in the patient. This prospect has been advanced and broadened into other diseases, such as cancer (Vile et al. 2000; Kerr 2003; McNeish et al. 2004), infectious diseases (Bunnell & Morgan 1998) and wound healing (Cutroneo 2003). In such patients the gene is delivered to enhance the natural protein production, amend the expression of the defective genes, kill or stop the growth of cells (tumor) and enhance cell proliferation in some cases. Lastly, viruses and viral vectors are immunogenic and hence initiate immune responds which could be exploited for DNA vaccines (Liu 2003).

The first clinical success of gene therapy announced in 2000 (Cavazzana-Calvo et al. 2000) required 10 years for development in clinical trials (Blaese et al. 1995). Since 1989, there are not many reports of successful clinical trials for gene therapy. The bottleneck of this difficulty is the toxicity, effectiveness and controllability of both viral and non-viral (synthetic) vectors (Verma & Somia 1997). The strengths and weaknesses of viral and synthetic vectors are listed in Table 1.2; we have divided the synthetic vectors into lipoplexes (lipidic-based systems) and polyplexes (polymer-based systems). Toxicity and safety issues are the key features for viral vectors while it is the effectiveness of delivery for synthetic vectors.

The main challenges for gene therapy are addressed as the following: i) DNA is biological material and it is readily degraded outside the cell nucleus; ii) the DNA molecule is large, and thus it is recognized and cleared from bloodstream by
reticuloendothelial systems (in liver and spleen); iii) DNA cannot penetrate tissues and it is too fragile to travel, _per se_, to target cells; iv) the negative charges of the DNA repels the negative charged cellular membrane preventing internalization; v) DNA is possibly degraded by lysosomes and may not reach the nucleus; vi) macromolecular crowding in the cytosol is likely to hinder the migration of DNA molecules towards the cell nucleus and vii) there is the difficulty of DNA constructs crossing the nuclear membrane.

**Table 1.2** Strength and weakness of viral and synthetic vectors

<table>
<thead>
<tr>
<th>Vectors</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viral vectors:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>retrovirus, lentivirus, adenovirus, adeno-associated virus, herpes simplex virus, pox virus</td>
<td>Efficient gene transfer Entering both dividing and non-dividing cells</td>
<td>Limited insert size (8-30 kb) Difficult to reproduce and scale up Possible to revert to a wild-type Induces dangerous immune reactions Silent oncogen Safety issues</td>
</tr>
<tr>
<td><strong>Synthetic vectors:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipoplexes - liposomes, lipidic base carrier systems</td>
<td>Available for multifunctional modification</td>
<td>Lower gene transfer Difficult to reproduce Toxicity concern Stability based on colloidal system (systemic administration)</td>
</tr>
<tr>
<td><strong>Synthetic vectors:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyplexes - polymers containing primary, secondary, tertiary and quaternary amines (can be linear, branch and dendritic structures)</td>
<td>Biocompatible Available for multifunctional modification Stable and robust manufacturing Facile manufacturing</td>
<td>Poor gene transfection efficiency but comparable to lipoplexes</td>
</tr>
</tbody>
</table>

*This table is adapted from Pack et al 2005

1.5 Biological and cellular barriers for DNA delivery

These reviews will be focusing on synthetic vectors. As mentioned in the last section on the limitation of DNA molecules, the vectors have to provide all the right circumstances to facilitate a thriving gene transfer. A simple concept is to imitate the natural environment of the DNA inside the nucleus where it is packed and its negative charges are enclosed. Various materials have been sought for their ability to condense
DNA, presented in two main categories: lipoplexes and polyplexes as shown in Table 1.2 (Pack et al. 2005). The vectors should overcome various impediments encompassing extracellular and intracellular barriers (Table 1.3).

**Table 1.3** An ideal vector to overcome extracellular and intracellular barriers

<table>
<thead>
<tr>
<th>Design criteria for the ideal synthetic vectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>• DNA protection</td>
</tr>
<tr>
<td>• DNA condensation</td>
</tr>
<tr>
<td>• Serum independent</td>
</tr>
<tr>
<td>• Targetability to specific cell types</td>
</tr>
<tr>
<td>• Reproducibility and easy to fabricate</td>
</tr>
<tr>
<td>• Robust and stable systems</td>
</tr>
<tr>
<td>• Ability to internalize both dividing and non-dividing</td>
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<tr>
<td>• Ability to escape from endosomes</td>
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<td>• Efficient disassembly</td>
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<tr>
<td>• Nuclear internalization</td>
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<tr>
<td>• Safety: non-toxic, non-immunogenic, non-oncogenic and non-pathogenic</td>
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**Modified from (Zauner et al. 1998; Brown et al. 2001; Pack et al. 2005)**

1.5.1 Extracellular barriers

From the administration sites to the target cells the vector-DNA complexes need to be primary targeted and protected from the harsh environment and interaction with other proteins in the circulatory system or tissues. The techniques use to overcome these obstacles include the physical administration of the complexes into cell nuclei of the target cells by gene gun and electroporation (Wells et al. 2000), direct administration to pathological sites or close to the sites (Nabel et al. 1996; Porteous et al. 2007) and ligand targeting (Ogris et al. 1999).

To overcome any unwanted interaction, the systems could be readily evaluated in the presence and absence of serum in transfecting medium in culture. Neutral and negative charges of complexes at physiological pH could induce the interaction with other proteins and aggregation of the complexes whereas positively charged constructs may remain stable in the circulatory systems. The aggregation leads to recognition of reticuloendothelial systems and end up with inefficient delivery (Dash et al. 1999).
Lai and Zanten have concentrated on kinetics of DNA condensation using time-resolved multiangle laser light scattering (TR-MALLS) and they demonstrated that the polyplexes between poly-l-lysine and DNA at certain mass molar ratios may undergo secondary aggregation several hours after the primary polyplexes were formed (Lai & van Zanten 2001). This implied that aggregates may be formed in a time dependent manner after administration of the polyplexes (Lai and Zanten, 2001). Surface engineering of the complexes with hydrophobic polymers (PEG, HPMA (Ogris et al. 1999;Toncheva et al. 1998), oligosaccharides (Wang et al. 2001) and proteins (Kircheis et al. 2001) may resolve this problem.

1.5.2 Intracellular barriers

After the complexes arrive at their target cells, they have to be internalized. Most of the polyplexes provide cell-targeting ability and such concepts can be achieved by using receptor-mediated endocytosis. Classical example is performed by Wu and Wu (Wu & Wu 1987;Wu & Wu 1988;Wu et al. 1989;Wu et al. 1991) using asialoorosomucoid proteins to enhance transfection efficiency of a polylsine polymer to target their receptors, asialoglycoprotein receptors, on the cell membrane of hepatocytes. Other examples include folate (Leamon et al. 1999;Lee & Huang 1996), transferrin (Cotten et al. 1990), epidermal growth factors (EGF) (Schaffer et al. 1997) and intregrin-binding sequences (Harbottle et al. 1998).

Most polyplexes particularly cationic are likely to be internalized by endocytosis and the summary uptake process is depicted in Figure 1.7A. The untreated polyplexes possibly bind to cell surfaces due to electrostatic attraction and might be taken up through adsorptive endocytosis (Mislick & Baldeschwieler 1996). When the endosomes transform into lysosomes the pH inside the vesicles drops to about 5-6, indicating an acidic environment which is fatal to DNA. The DNA therefore needs to be escape from such vesicles. Combat mechanisms for escaping involve the addition of endosomolytic agents (chloroquine), vector conjugated with endosomolytic peptides (Zauner et al. 1998) or fusogenic peptides (Lee et al. 2001). Particular types of polymers (PAMAM, PEI), containing large amount of secondary and tertiary amines and having pKa's between physiological and lysosomal pH, exhibit a “proton-
sponge” effect (Figure 1.7B) to enhance escape (Haensler & Szoka, Jr. 1993; Behr 1997; Sonawane et al. 2003). The pH inside endosome allows protonation of the polymer and aids the influx of counter-ions to buffer the system, causing an increase in ionic concentration. The water molecules are taken in to reduce such high concentration activating osmotic swelling and disruption of the endosomes.

Figure 1.7  Schematic diagrams summarising intracellular barriers (A) and a “proton sponge” effect (B) of polyplexes. The polyplexes are usually taken up by endocytosis. Some are able to escape from endosomes and traverse to cell nucleus whereas some remain and are degraded in lysosomes. Particular types of polymers display the proton sponge effect to escape from endosomes, as described in the text (Figure is adopted from (Pack et al. 2005)).

Migration of the DNA toward cell nucleus is the topic which has received some attention; this step may be the rate limiting step for efficient gene transfer. Cytosol
possesses a macromolecularly crowded environment which is quite different from dilute solution. Polyplexes may traverse through endosomes along the microtubules to reach the cell nucleus (Suh et al. 2003) or they may simply diffuse through such crowded conditions (Luby-Phelps et al. 1987; Lukacs et al. 2000) after escaping from endosomes. Diffusion inside the cytosol is size dependent and the DNA is commonly degraded in the cytosol by cytosol nucleases (Lechardeur et al. 1999). The exact road map of the DNA and synthetic vectors inside cytosol has remained unclear.

Cells contain various types of macromolecules in a submicroscopic organisation indicating that the physiochemical properties of the cell cytoplasm are different from dilute solution. In addition, the cell cytoplasm is a non-homogeneous, non-Newtonian and non-isotropic system pervaded by a network of self-assembling polymeric protein fibers known collectively as the cytoskeleton (Luby-Phelps et al. 1987). This leads to "macromolecular crowding" which describes the intracellular environment as a physiological medium physically occupied by a high macromolecular content as a significant proportion of the total cytosol volume (Ellis 2001a; Ellis 2001b). Although there is no single molecule at a high concentration, taken together the total macromolecular concentration is about 50 - 400 mg/ml (Fulton 1982; Lanni et al. 1985; Cayley et al. 1991) which engaged 5 - 40% of the total volume of the cells (Ellis & Minton 2003). The scaled drawings of the cytoplasm in which macromolecular crowding can be fully appreciated are presented in Figure 1.8.

Because two macromolecules can not occupy the same space at the same time, the crowding effect and the excluded volume effect are originated. "Crowding is more precisely termed the excluded volume effect, which emphasises the fact that it is a purely physical non-specific effect originating from steric repulsion" according to Ellis (2001). For each macromolecular species inside cells, the effective concentration, or thermodynamic activity is thus greater than its actual concentration and this difference has kinetic and thermodynamic consequences for the properties of that macromolecule.
Macromolecular crowding is simply referred to as excluded volume effects or non-specific steric effects, on a test particle (macromolecule) due to the presence of a background of other particles (or macromolecules), often present at high total concentration. A crowded solution refers to a solution containing a high total concentration of macromolecules. The effect of exclusion volume is to play a prominent role for larger molecules over smaller molecules, as can be seen in Figure 1.9.

Macromolecular crowding has significant effects on the thermodynamics of the cell and strongly influences diffusion processes inside the cell (Luby-Phelps et al. 1987). The effects on the thermodynamics of the cell are, for example, that it induces the tendency to protein association (Rivas et al. 1999), promotes protein refolding and aggregation (Van den Berg et al. 1999; Van den Berg et al. 2000). A number of reviews on this effect, including quantitative and qualitative thermodynamic aspects of macromolecular crowding, are presented elsewhere (Zimmerman & Minton 1993; Zimmerman 1993; Hall & Minton 2003). In this manuscript we will focus on the effect of macromolecular crowding on diffusion processes inside the cell and the contribution of this in intracellular transportation.
Figure 1.9 The volume exclusion effect. A small molecule is free to occupy the entire volume in between the black obstacles (i.e. the white and grey areas). A molecule of comparable size to the obstacles is much more limited in the volume it can occupy: the centre of such a molecule is limited to the white areas only (Schnell & Turner 2004).

In dilute solution the mobility of particles or molecules can be described by a random walk (Zimmerman 1993) and the diffusion of the particle can be calculated from

\[(r^2) = 2dD^0 t \] ...................................(1.1)

where \((r^2)\) is the squared displacement of the position observed after elapsed time \(t\), average over many observations; \(d\) is the dimensionality. If the directions of the steps of a random walk are delimited to a line, e.g. to just back and forth, it is a one-dimensional random walk. If they are restricted to a plane, it is a two-dimensional random walk. \(D^0\) is the intrinsic diffusion coefficient of species \(i\), which is given by the Einstein relation:

\[D^0_i = \frac{kT}{\eta} \] ...................................(1.2)

where \(\eta\) represents the frictional coefficient of an isolated tracer particle of species \(i\) in a solvent of given viscosity and \(k\) is Boltzmann’s constant. In the absence of a macroscopic gradient of background molecules the diffusion coefficient of particles becomes self-diffusion because the tracer and the background species are identical.
The effect of background molecules is, however, extremely complex to analyze (Zimmerman 1993). From equation 1.1 the diffusion coefficients (D) inside the cell could be decreased up to 10 times of that in water. Since the average time a molecule takes to move a certain distance is proportional to $D^{-1}$, hence if the D value is reduced 10 times, it will take 10 times as long for a molecule to travel in the same distance. The diffusion of large particles or macromolecules will be hindered more than that of smaller particles or molecules.

The final target of the polyplexes is the cell nucleus and entry to the nuclear compartment is controlled by nuclear pore complexes (NPC) in the nuclear membrane. More detail can be found in Chapter V of the thesis. Regarding the large size of polyplexes, they are not able to passively diffuse through the NPC. Various ligands have been exploited for this secondary targeting such as nuclear localisation signals (NLS) (Chan & Jans 1999; Chan et al. 2000).

1.6 Lysine-based dendrimers and dendrons

The chirality of amino acid molecules confers the possibility that amino acid molecules can be constructed as “peptide dendrimers” increasing their biocompatibilities and diversity (Jang & Kataoka 2005). In fact the specific folding of amino acid branch units may facilitate the presence of the unique three-dimensional architecture of peptide dendrimers. Peptide dendrimers have been employed as drug and gene carriers, artificial proteins and catalysts. Polylsine dendrimers are one of peptide dendrimers derived from repeated units of amino acid derivatives. There are reasons for the construction of polylsine-based dendrimers as gene carrier system. Firstly, linear polylsine and polylsine dendrimers have been explored for DNA delivery for more than 20 years because of the marked presence of lysines in histone molecules inside the cell nucleus which play crucial role in DNA condensation and transcription (Männistö et al. 2002). Polylsine per se has the ability to condense DNA molecules and display high gene transfer in vitro (Zauner et al. 1998). Secondly, polylsine is also commercially available in wide range of molecular weights and it can be simply degraded by cells. Thirdly, lysines possess epsilon amino acids, which become positively charged at physiological pH and thus they can interact with other
negatively charged molecules such as DNA. This interaction was exclusively investigated (Laemmli 1975; Reich et al. 1990; Granados & Bello 1981; Chang et al. 1973; Shapiro et al. 1969). Moreover, the epsilon amino groups of lysines also offer a great chance for lysine to be covalently attached to other ligand molecules. Finally, lysines contain two amino groups which can serve as branch units for dendritic architecture.

In comparison to other cationic dendrimers such as PAMAM and PPI dendrimers, polylysine (PLL) dendrimers possess positive surface charges displaying an ability to condense DNA molecules with lower toxicity effect than the dendriplexes obtained from PAMAM and PPI dendrimers (Okuda et al. 2003; Ohsaki et al. 2002; Plank et al. 1996). Ohsaki et al. (2002) demonstrated that mono-dispersed dendritic poly-l-lysine (DPK) having hexamethylenediamine as a core show high transfection efficiencies without significant toxicity. They also found that the PAMAM dendrimer showed maximum efficiency at generation 6 - containing 192 amino groups- whereas their dendritic poly-l-lysine (KG6) showed higher efficiency with 128 amino groups indicating its optimal generation for gene transfection. Okuda et al. (2003) speculated on time dependent dendriplex formation of dendritic PLL and DNA and the results of aggregation of dendriplexes with more than 1 μm in diameter which contained various small particles of the complexes ranging from 50-200 nm in diameter as a major species. The same group substituted the terminal lysines with arginines (KGR6) or histidines (KGH6) and the results indicated higher transfection of KGR6 while the KGH6 did not transfect (Okuda et al. 2004).

The transfection efficacy of the linear, grafted and dendritic poly-l-lysine (G3, G5) was compared by Männistö et al. (2002). DNA was condensed by most types of PLL except G3-PLL dendrimer, and the linear PLL exhibited higher transfection efficiency than PLL dendrimers. The G5-PLL dendrimer illustrated high DNA condensation but presented low transfection efficiency. The pegylation increased transfection efficiency of linear PLL and grafted PLL but not dendritic PLL. All architectures, both pegylated and non pegylated PLL, showed lower transfection ability by comparison with the positive control (PEI), although the transfection can be enhanced by pegylation of
linear PLL (charge ratio 4:1(+/-)). The reason for low transfection of dendritic poly-l-lysine may be explained by the interaction between the complexes with glycoaminoglycans (GAGs) abundantly found in the extracellular matrix and cell surface may result in the release of the DNA from the complexes before they were taken up (Ruponen et al. 1999; Männistö et al. 2002). In addition PLL, per se may possess poor ability to escape from endosomes after endocytosis (Zauner et al. 1998).

Many methods have been addressed for this problem, for instance, the addition of either chloroquine (lysosomotropic agents) or glycerol (endosomal membrane enhancer) during transfection process as well as the combination transfection of PLL with lipid-based transfecting agents (Trubetskoy et al. 1992). The addition of inactivated adenovirus to PLL-DNA complexes was also studied and resulted in transfection efficiency up to 2000 fold over controls (Curiel et al. 1991; Wagner et al. 1992; Cotten et al. 1992). The PLL was conjugated with membrane-disruption peptides or receptor-mediated uptake peptides to improve transfection ability. An excellent review on these endeavors can be found in Zaunner et al. (1998) and Pack et al. (2005).

The attachment of other functional groups on the primary amino groups of lysine dendrimer makes various applications available. For instance, a calixarene-modified PLL dendrimer is found to exhibit as a multivalent host molecule which may serve as a nanoreceptor with a strong affinity for substrates (Xu et al. 2003). Multiple antigen peptides (MAPs) provide other examples of peptide dendrimers (Sadler & Tam 2002) synthesised by the attachment of the peptide sequences onto the amino surface groups of polylysine dendrimers providing a strong immunogenic effect to dendritic molecules.

1.6.1 Self-assembly of dendrimers and dendrons

In Florence’s research group, the first interest in dendrimers was placed on the secondary structure of a homologous family of polylysine-based dendrons and dendrimers, which were cationic, lipic and amphipathic in nature (Al-Jamal et al. 2005). An alteration in solubility, molecular shape and solution or suspension
behavior can be found by modifying surface groups of the dendrimers and these phenomena was, first, evidenced by Sakthivel et al (1998). The 4th generation of lipidic symmetrical dendrimers formed asymmetric fibre-like structures in aqueous media as shown in Figure 1.10.

The assembly of dendrons may be explained by the effects of hydrogen bonding (Zimmerman et al. 1996), hydrophobic interaction (Percec et al. 2001) as well as electrostatic interactions (Blanzat et al. 2002; Tomioka et al. 1998). A synopsis of the self-assembly structures of the lysine-based dendrons and dendrimers related to their applications obtained from Florence’s group is demonstrated in Figure 1.11. The hydrophobic symmetric dendrimers both generation 5 and 6 with different lipidic chain lengths could form monolayer (Singh 2005) as shown in Figure 1.12. The formation of monolayers was assumed to be dependent on hydophobicity of the lipidic chains in dendrimer molecules. An increase in length of the lipidic chain could result in an increase in contact angle (>90°) indicating smaller part of the dendrimer would embed in the aqueous subphase (at the air/water interface). The results from π/A-isotherms reflected the area/dendrimer molecule which increase with the increase in dendrimer generation and lipidic chain length (Sakthivel et al. 1998; Singh 2005). Unlike with rigid spheres, this method may not always be consistent because of the elasticity or collapsibility of the dendrimer molecules (Al-Jamal et al. 2005). Dendrimer flexibility emerged from the change in dimensions of dendrimer molecules according to the pH of their suspending medium (Kim & Bruening 2003).
Figure 1.10  Self-assembly models of the lipophilic dendrimer in aqueous media (A): the dendrimers typically exhibit as microtube with 140-200 nm length and 24 nm in diameter (B). This microtube is possibly associating as a bundle (C) (Sakthivel et al., 1998).

Figure 1.11  A summary of the primary (peach), secondary (blue) and tertiary (green) structures of the polylysine dendrimers and dendrons relating to their applications. In state of aggregation the dendrimers and dendrons are found as monolayer, nanoparticles, dendrisomes, dendriplexes and dendrimer constructs encompassing adsorption of the dendrimer onto other particles (modified from Al-Jamal et al. 2005).
1.6.2 Dendrimer-derived nanoparticles

Dendrimers possessing partial or entire hydrophobic groups may appear as aggregates. The controllable nanoparticles formulated from various lipophilic chain lengths of dendrimers (G5-6) can be achieved by precipitation method (Quintanar-Guerrero et al. 1998) with and without surfactant addition (Singh & Florence 2005). The increase in generation resulted in an increase in diameter whereas the increase of lipidic length affected no influence on particle diameters obtained from the 5th generation. This effect was found to be variable in the aggregates of the 6th generation dendrimers as demonstrated in Figure 1.13. By using pyrene - a hydrophobic fluorescent probe – the longer the lipidic chain length and the increase in dendrimer concentration caused the denser form of aggregates with a more hydrophobic interior.

1.6.3 Micellar aggregation

Amphipathic dendrons, (C_{14})_{3}\text{Lys}_{7}(\text{NH}_2)_8 with three lipidic chains and 8 amino groups (Figure 1.14) were found to form micelle-like structures (Figure 1.15) with diameters between 20-30 nm. The aggregates became larger, 30 nm in diameter, in the presence of 0.1% NaCl whereas a smaller aggregate, 14 nm in diameter, was found in distilled water. The homogeneous aggregation of dimeric dendrons was observed in the dendrons containing one alkyl lipidic chain. Other dendrons in this series with the higher number of lipidic chains for instance (C_{14})_{2}\text{Lys}_{7}(\text{NH}_2)_8, (C_{18})_{3}\text{Lys}_{7}(\text{NH}_2)_8 and (C_{18})_{3}\text{Lys}_{15}(\text{NH}_2)_{16} were also found to form micellar aggregates. It was noticed that
when the lipidic chain length was increased from $C_{14}$ to $C_{18}$ that aggregation occurred. As the lipidic chains of the dendron are assumed to play the same role as the lipidic tail in traditional surfactants the aggregation therefore could be predicted from surface free energy (Al-Jamal et al. 2005).

Figure 1.13  Effect of particle generation and lipidic chain length on the apparent particle diameters of the aggregates obtained from the 5th (dendrimer 1-3) and the 6th generations (dendrimer 4-6) of amphipathic dendrimers. The 5th generation with $C_{4}$, $C_{10}$ and $C_{12}$ lipidic chains are dendrimer I, II and II and the 6th generations with $C_{4}$, $C_{10}$ and $C_{12}$ lipidic chains are dendrimer IV, V and VI, respectively. The increase in generation initiates the increase in diameter of the aggregates. The increase in the length of lipidic chains shows no effect on the apparent diameter at the lower generation while the opposite is found in at higher generation (Singh & Florence 2005).
Figure 1.14 Molecular structures of the amphipathic dendrons bearing different number of lipidic chains (n): the structure is composed of hydrophobic head groups of lysine branching units and hydrophobic tail (adopted from Al-Jamal et al. 2005).

Figure 1.15 Micellar aggregations of the amphipathic dendrons, \((C_{14})_1\text{Lys}_7\text{(NH}_2)_8\), in the presence (A) or absence (B) of 0.1% NaCl. The size of the aggregates is increased in the presence of NaCl (Ramaswamy & Florence 2005).
1.6.4 Dendrisomes

A novel cationic lipidic polylysine dendron, \((C_{14})_3\text{Lys}_7(NH_2)_8\), Figure 1.16 was found to self-assemble in water forming structures named “dendrisomes” which capable of encapsulating small drug molecules, penicillin G (Al-Jamal et al. 2003b; Al-Jamal et al. 2005). The dendrisomes were prepared by the reverse-phase evaporation method (REV) in the presence and absence of cholesterol. The alteration of vesicle morphology and diameter depended on the content of cholesterol. The average hydrodynamic diameter of the dendrisomes without cholesterol was found to be 311± 8 nm whereas that was increased to 557 ± 13 nm in the presence of cholesterol. The irregularity and the shape of the dendrisomes increased with the increase in cholesterol content whereas a smaller effect was found on the zeta potential. In comparison to the liposomes prepared by the same method, the dendrisomes exhibited higher percentage of drug encapsulation; the percentage of drug encapsulation in the cholesterol-free dendrisomes, 6.15%, higher than that of the dendrisomes with cholesterol, 4.4.74%. This was due, it was suggested, to the substitution of the dendron in the bilayers with cholesterol (Al-Jamal et al. 2003b).

1.6.5 Dendron/dendrimer constructs

Sakthivel and Florence (2003) demonstrated the ability of the amphipathic dendrons, \((C_{14})_3\text{Lys}_7(NH_2)_8\), \((C_{14})_3\text{Lys}_{15}(NH_2)_8\) and \((C_{14})_3\text{Lys}_{31}(NH_2)_{32}\), to adsorb on surface of polystyrene nanoparticles, 200nm in diameter, via hydrophobic interactions. The experiment was performed in phosphate buffer saline (PBS). The augmentation of the generation and hydrophobicity resulted in the reduction of the adsorption of the dendrons (Sakthivel & Florence 2003). As the adsorption generally increased with the decrease in dendrimer concentration, it is presumed that dendrons may form monolayer on the particle and area/molecule of the dendrons thus was elucidated. Similar studies on the adsorption of similar dendron species on liposomes have also been reported (Purohit et al. 2001).

1.6.6 Dendrimer stabilized emulsions

Amphipathic dendrimers also behave as emulsifiers as they contained hydrophilic head and hydrophobic tail. Apart from lysine based dendrimers, other types of
dendrimer such as PAMAM (Yi et al. 2004), polypropyleneimine dendrimers (Xu & Ford 2002) also act as emulsifying agents. Both dendrimers benefited the production of monodispersed of polystyrene with particle size smaller than 100 nm.

Figure 1.16 Molecular modeling of the “dendrisomes” formulated using the REV method: the lipidic dendron (A) at air/water interface (B), in vacuum (C) and in polar environment (D). The dendrons are likely to aggregate in vacuo (C) while it arranges its aliphatic alkyl chains toward the air at the water surface (B). Structure of the dendrisomes is magnified in E; the vesicle is constructed from bilayers of dendron molecules facing their apolar aliphatic alkyl chains to each other. The thickness of the dendrisome “wall” is found to be between 6.6-10 nm which is thicker than that of liposomes formulated by the same method (Al-Jamal et al., 2005).
1.6.7 Dendriplexes and applications for DNA delivery

The second interest of our group is on the formulation of the dendriplexes and their application in gene delivery. Complexes formed between the dendrimers/dendrons and the DNA have been coined “dendriplexes” (Ramaswamy et al. 2003a); these have potential application in gene delivery. Dendriplexes obtained from lipidic poly-l-lysine dendrimers exhibited higher gene transfection than cationic liposomes (Surovoy et al. 1998). Various lysine-based dendrons and dendrimers were synthesised in our groups and their ability to condense the DNA and their transfection efficiency have been evaluated (Toth et al. 1999; Bayele et al. 2005; Shah et al. 2000).

Toth et al. (1999) synthesised a series of asymmetric amphipathic dendrons consisting of three (C_{10}) chains. The chemical structures of the dendrons in this series are based on structures in Figure 1.14 except that the \( C_{14} \) is replaced with \( C_{10} \) which developed further by the changes in the number (8, 16, 32 amino groups) and types of amino acids (Ornithine or Lysine) in the hydrophilic head, as well as the attachment of sugar units and a nuclear localisation peptide sequence (NLP) to be able to achieve gene transfer. The results indicated that all dendrons could condense DNA and facilitate transfection of the DNA with insignificant toxicity excepting the ornithine-based dendrons, containing 8 and 16 amino groups in their hydrophilic heads, which have very low solubility. The increase in the number of amino acids in the hydrophilic head groups of dendrons generally resulted in the increase in transfection efficiency in Cos-7 cells but the opposite was found in the dendrons conjugated with sugars. The optimal amount of the DNA and the charge ratios of dendriplexes have been evaluated. The transfection is serum-independent and the addition of other cationic compounds. The maximum and minimum transfection efficacies were found in \((C_{10})_3\text{Lys}_{15}(\text{NH}_2)_{16}\), and the dendrons with NLP-peptide conjugated.

The dendrons containing ornithine branch units showed lower transfection efficiency than analogues with lysine branch units indicating the difference in the linker length affects the transfection (Tote et al. 1999). Unfortunately, attachment of sugar molecules to the dendrons did not increase transfection, neither did the introduction of the NLP sequence. This may be due to steric effects perhaps after administration of
the polyplexes causing a lack of exposure of peptide on the surface. The same effect was also revealed in other cell lines (Shah et al., 2000). The toxicity level of the dendrons was evaluated and the results suggested that toxicity was reduced due to complexation with the DNA. The interaction between the DNA and the dendrons was assumed to be based on electrostatic interaction and this interaction seemed to be complete within 2-3 min after mixing. The complexes have show good DNA condensation and protection with the DNA release was found when the pH was increase to 11.

Recently, the influence of the lipidic chain length of the hydrophobic tail in the amphipathical asymmetric dendrons has been evaluated (Ramaswamy et al. 2003b). The ability to condense DNA was found to be not only governed by the electrostatic attraction of the lysines in the hydrophilic head group but also to be controlled by the hydrophobicity of the lipidic chain of the hydrophobic tail. The condensation was found in descending order: (C\(_18\))\(_3\)Lys\(_7\)(NH\(_2\))\(_8\) > (C\(_{14}\))\(_3\)Lys\(_7\)(NH\(_2\))\(_8\) > (C\(_{10}\))\(_3\)Lys\(_7\)(NH\(_2\))\(_8\) > Lys\(_7\)(NH\(_2\))\(_8\). This is confirmed by the significant reduction of the molar charge ratio acquired to the complete condensation of the DNA comparing with that of the non-lipidic dendron. The molecular modeling of the interaction between the dendrons and the DNA are presenting in Figure 1.17. This dynamic model permits the observation of the mobility of the dendrons toward the DNA backbone through hydrophobic interactions.

Similar types of polycationic dendrimers were studies the ability to form complexes with oligonucleotides and evaluated their transfecting ability in human retinal pigment epithelial cells (Wimmer et al. 2002). Another group has also studied amphiphilic dendrimers containing variable lengths of alkyl chains and a rigid tolane (diphenylethyne) core. The maximum binding ability and transfection efficacy was found in the dendrimers with three C\(_{12}\)-alkyl chains. This result has confirmed and underlined the influence of the hydrophobic interaction on ability to enhance the DNA condensation and transfection ability with low toxicity of these amphipathic vectors (Joester et al. 2003).
Ribeiro et al. (2005) has employed this concept and developed it further to achieve high DNA encapsulation and sustained release of DNA for vaccine delivery. The hybrid system in which dendriplexes were themselves encapsulated in PLGA nanoparticles offered higher DNA condensation and protection. Two types of dendrons, \((C_{10})_3\text{Lys}_{15}(NH_2)_{16}\) and \((C_{18})_3\text{Lys}_{15}(NH_2)_{16}\) were used; the dendriplexes were first formed and were encapsulated in PLGA particle by a double emulsification method (Ribeiro et al. 2005). Both dendrons provide high DNA condensation but different percentages of DNA encapsulations. By radio labelling the DNA, the maximal encapsulation of DNA was found in particles containing 10:1 (+/-) molar charge ratio of the C_{18}-dendron, 15.6% (w/w) whereas the encapsulation of naked DNA was 9.9% (w/w). The mean diameters were found to be 452 nm and 988 nm for the 10:1 PLGA-dendriplexes and the PLGA particles (containing naked DNA), respectively. The release profile demonstrated a burst release effect in the first 24h followed by the sustained released effect for more than 350h. About 55% of the DNA was released from the 10:1 PLGA-dendriplexes after 9 days while 27% was found for the PLGA particles.
For *in vivo* studies, this system has been employed for vaccine against anthrax (Ribeiro *et al.* 2007) which currently have problems on the unwanted side effects and the long length of immunization schedule. The dendriplex-PLGA particles serve as a carrier for gene initiating protective antigen (PA) of *Bacillus anthracis*. Three types of particles were formulated: PLGA-C_{18}-dendriplexes, PLGA-C_{0}-dendriplexes and PLGA-naked DNA system (control). Results illustrated that the significant increase of the anti-PA IgG antibody levels was obtained from the animals receiving the PLGA-C_{18}-dendriplexes particles in comparison to animals immunized with the PLGA-C_{0}-dendriplexes particles. The higher antibody level was found after the second booster. This might be explained by the sustained release of the DNA from the PLGA particles which facilitate the transfection of muscle cells aiding antigen presentation.

The interaction between the dendrimers with other types of proteins such as albumin (Purohit *et al.* 2003), heparin (Al-Jamal *et al.* 2003a) and cell cytoskeleton have also been investigated the details of which can be found in Chapter IV.

1.7 Aims and objectives of the thesis

There are areas where little work has been done but are essential to allow successful development of dendrimers for gene delivery. A road map of how the dendrimers delivery their passenger DNA to the nucleus remains a puzzle. The technique of fluorescent tracking has mainly been employed to fulfill these lacunae. However, some fluorescent tags perhaps alter the uptake partway of the parent molecules (e.g. Hoechst fluorescent dye enhances nuclear uptake (Fong *et al.* 2004), wheat germ agglutinin (WGA) inhibits nuclear uptake (Fischer *et al.* 1991;Zupan *et al.* 1996)). The ambiguous step might be manifested in nuclear internalising process whether the dendriplexes, or the degraded fluorescent tag, or the parent dendrimers/DNA are entering the cell nucleus. The exact pathway is still speculative. An intrinsically fluorescent lysine-based dendrimer has recently reported (Al-Jamal *et al.* 2006) and so no tags are need for the uptake study. This may help us to clarify what has been unclear.
The aim of this thesis majored on a study of the dynamic uptake of the lysine-based dendrimers and dendriplexes based on fluorescent microscopy, not employing fluorescent tags which possibly degrade but the intrinsic fluorescent properties of a dendrimers found in our laboratories. Simultaneously, the potential use of the dendrimers as carrier systems for DNA has been speculated as well as the interaction between the dendrimers and cellular compartments.

To achieve these aims the objectives of the works discussed in each chapter of this thesis were as follows:

- An intrinsically fluorescent lysine-based dendrimer has been synthesized by two methods: Fmoc and Boc solid phase peptide synthesis and the overall yields of both methods have been compared in Chapter II.

- The diffusion character of the dendrimer in comparison to nanoparticles has been explored in various types of media, for instance bulk solutions, polymer solutions and a gel developed to imitate cytosol vicinity and discussed in Chapter III.

- An ability of the dendrimer to condense the DNA forming dendriplexes and the transfection ability of this compound have been elucidated by comparison with other types of dendrimers in Chapter IV.

- The ability of the dendrimer to be used as nano-fluorescent probe has been evaluated and dynamic uptake of dendrimer and dendriplexes in living cells has been performed and presented in Chapter V.

- The ability to interact with cellular proteins, the interaction between the dendrimer and actin cytoskeleton has been observed in Chapter VI using various techniques such as fluorescent immunofluorescence, gel electrophoresis and biochemical assay. The capacity of the dendrimer as a cross-linker has also been evaluated.
Chapter II

DENDRIMER SYNTHESIS AND CHARACTERISATION

Summary
A 6th generation poly-lysine dendrimer, (Gly)(Lys)₆₀(NH₂)₆₄, was synthesized by Boc and Fmoc solid phase peptide synthesis (SPPS) and the compound was then characterised using mass spectrometry (MS), high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR). The Boc SPPS provided a higher yield of dendrimer, 64.10%, than did the Fmoc SPPS (yield 30.04%). The results from MS confirmed the molecular weight of the dendrimer synthesised by Boc SPPS at 8149 Da (theoretical value of WM is 8149.05 Da). The structure of the compound was confirmed by ¹H NMR. The chromatograms from reverse phase-HPLC (RP-HPLC) suggested that Boc SPPS should be used as the method for synthesising this type of peptide dendrimer because it provided high yield of product with only minor impurity. The product of dendrimer acquired from Boc SPPS was, therefore, employed in all studies presents in this thesis.
2.1 Introduction

Since they were first introduced in 1978 by Vögtle and co-workers, dendrimers have been employed in various applications as mentioned in Chapter I. A multiplicity of dendrimers has been synthesized. In our group, a wide range of lysine-based dendrimers and dendrons have been synthesized (Sakthivel et al. 1998; Al-Jamal et al. 2005) and their prospects as drug carrier systems (Al-Jamal et al. 2003b; Singh 2005), gene carrier systems (Toth et al. 1999; Bayele et al. 2005; Ramaswamy et al. 2003a; Ribeiro et al. 2005) and vaccine delivery vectors (Ribeiro et al. 2007) were carried out. Their interaction with other proteins (Purohit et al. 2003) and liposomes has also been investigated (Purohit et al. 2001). Among all, a symmetrically poly-lysine dendrimers with glycine amino acid core was serendipitously found to have intrinsic fluorescence without containing any fluorophore, yet possessing sufficient fluorescence intensity to be detected at low-concentrations, avoiding the need for fluorescent labelling (Al-Jamal et al. 2006). We have used this compound as “a nanoprobe” for dynamic mobility of the dendrimer per se inside cells (Chapter V). Implicit in this particular step is utmost important to achieve high gene expression and effective drug delivery.

In this chapter the synthesis of this 6th generation poly-lysine dendrimer with a glycine amino acid core is described. We used a divergent synthetic strategy with solid phase peptide synthesis (SPPS) using Boc- and Fmoc-methodology. The compounds were purified by dialysis and then characterised using NMR, MS and HPLC. The potential for use of this dendrimer as a nanoprobe has been confirmed by a spectrofluorimetric method (see Chapter V).

2.2 Dendrimer synthesis

Dendrimers have hitherto been synthesized using two main strategies: divergent and convergent. The divergent strategy was the first method emerged simultaneously since the dendrimer was introduced and pioneered by Vögtle and co-workers (1978). By this strategy, the dendrimer is constructed from the dendrimer core toward its periphery, generation by generation, as illustrated in Figure 2.1. Although this
strategy benefits fully through control with a high yield of low generation dendrimers, when applied to higher generations, reports on the incidence of the incomplete dendrimer were obtained. Notably, in this strategy the contamination of byproducts having similar molecular weights to the desired dendrimer is a great concern which, however, can be reduced by an alteration of the amount and type of the coupling reagents (Kate & Albericio 2000).

The convergent strategy was established by Fréchet and Hawker in 1990 and was claimed to overcome the difficulties of the divergent approach. The dendrimer constructed by this strategy is built from its periphery toward the dendrimer core (Hawker & Fréchet 1990). As a consequence, the number of the reaction in each step is reduced to two and the byproduct are more readily discriminated and eradicated owing to the larger mass difference between the desired dendrimer and its byproduct. The detriments of this strategy are that the lower yield and the slower reaction due to the steric crowding of the periphery which may reduce reactivity of the reactive group at the focal point during the coupling process. In this work the lysine-based dendrimer was synthesis using divergent strategy as optimized in our laboratory (Sakthivel et al. 1998).
Figure 2.1  Dendrimer synthetic strategies: divergent approach, the dendrimer is built from its multifunctional core and built up monomer layer by monomer layer (adopted from Dufès, et al. 2005). The core molecule is reacted to monomer molecules containing two (or more) protecting groups giving the first generation dendrimer. The protecting group at the periphery is subsequently deprotected and activated, and thus it is ready to react with a new monomers layer. The reaction sequence is reiterated with the next generation of monomers until achieve the desired product. For the convergent approach the dendrimer is also synthesized layer after layer, however, starting from the end groups to the core. First, two (or more) peripheral branch subunits are reacted with a single joining unit (focal point) which has two (or more) corresponding active sites and a distal inactive site creating the first outer layer. A new larger branch subunit is again reacted with a focal point until reaching target size. Finally, a core molecule is attached at the focal point to yield the dendrimer.
2.2.1 Solid Phase Peptide Synthesis (SPPS)

Since the desired dendrimer in this work is 'a peptide dendrimer' or a lysine-based dendrimer, solid phase peptide synthesis (SPPS) is implemented for dendrimer synthesis. Peptide synthesis entails the formation of amide bond between a carboxyl group of one amino acid and an amino group of another amino acid, which usually requires a large amount of energy. Thus the direct condensation can be accomplished at high temperature (160-180 °C), which is normally incompatible with the existence of other functionalities (Montalbetti & Falque 2005). Consequently, the activation of either the carboxyl or amino terminal is a requisite. The activation of carboxylate group is the most common method used in the peptide coupling process, which involves an attachment of a good leaving group (Y) to the acyl carbon of the acid causing it becomes more electro-positive and allows nucleophillic attack by the amino group of another amino acid (Figure 2.2). An intermediate formation of tetrahedral structure is finally stabilized by elimination of the good leaving group establishing an amide bond.

Classically, peptide synthesis was carried out using solution peptide synthesis (SPS), which required a great deal of expertise and experience. A small amount of protein had been synthesized because SPS was a time consuming process. This technique also showed downsides of low overall yield, high contamination of side products and high incidence of racemisation. The SPPS is a high-profile method above the SPS because the synthesis can be automated as it is a simple straight-forward process and provides high yield of products. The SPPS was first established by Merrifield in 1962 with a full publication in 1963 (Merrifield 1962; Merrifield 1963). The 1963 paper explained the synthesis of the tetrapeptide, L-leucine-L-alanylglucyl-L-valine involved the stepwise addition of benzyloxycarbonyl-protected amino acids to a growing peptide chain which covalently bound to a solid resin particles. Although, the synthesis was undoubtedly successful, the coupling reaction was not reached 100% yield and there was the contamination of the shorter peptides. The method was later modified and improved in 1964 by replacing benzyloxycarbonyl-protected amino acids with the stronger acid-labile t-butyloxycarbonyl-protected amino acids resulted in the highly
purified form of bradykinin (Merrifield 1964). Since then the SPPS has been a cornerstone in peptide chemistry.

Figure 2.2 The principle of peptide bond formation

In principle, the chemical reaction of peptide assembly using SPPS, illustrated in Figure 2.3, is actually occurring inside and on the solid support while the carboxylic terminal of the peptide is anchored onto the solid support throughout the coupling process. The solid support is a gel of resin beads which swell but not dissolve in the particular medium, N,N-dimethylformamide (DMF). The peptide grows in one direction from the carboxylic terminal (C-terminal) toward the amine terminal (N-terminal) in stepwise manner. When the first protected α-amino acid and side-chain protected amino acid residues is added (1), the coupling reaction is conducted (2). As a result, the racemisation can be minimized, and the surplus reactants and byproduct can be easily separated from the desired peptide by sufficient washing and filtration. After the complete coupling (3), either acid-labile tert-butoxycarbonyl (Boc) or base-labile 9-fluorenylmethoxycarbonyl (Fmoc) of the first α-amino acid is deprotected (4) and the second protected amino acid is added. The complete coupling (3) and deprotection (4) are routinely monitored for successful peptide synthesis. The coupling and deprotection processes are repeated until the desired peptide is achieved. The protecting groups and side chain protecting groups are simultaneously cleaved from the solid support (5) using a method depending on type of selected solid support.
Selection of α-amino protecting and side chain protecting groups

Loading of protected amino acid

Activation & coupling

Monitoring complete coupling

Deprotection

Cleavage from solid

Desired dendrimer

Figure 2.3 A simply stepwise process of dendrimer synthesis using SPPS. The process starts from 1 the selection of solid support which restricted by Boc or Fmoc methodology and this must correspond with 2 the selection of protecting groups of α-amino acid and its side chain protecting groups. After a swelling process of the solid support (resin) the protected amino acid is loaded (3) and coupling reactions begin (4). When the completion of the first coupling is scrutinized (5), the deprotection (6) is carried on and monitored (7). The next generation of the protected amino acid is added, and whole process of coupling reaction followed by the deprotection is repeated (4-7) until the desired dendrimer is achieved. Finally the desired dendrimer is cleaved from the solid support (8) using a method dependent on the solid support selected.

2.2.1.1 Solid support (resin)

The selection of solid support is an important step to achieve effective dendrimer synthesis. Ideally, solid supports have to be stable and their size and shape have to ease manipulation and rapid filtration (Kate & Albericio 2000). Secondly, the solid support has to be inert to all reagents and solvents used throughout the synthesis process. Thirdly, it has to swell amply in the solvent used for synthesis and consent all reagents to penetrate promptly throughout the particles of the solid support. Lastly, it has to be willingly modified to permit attachment of the first entity by a covalent bond.

In Merrifield’s system, a chloromethylated copolymer of polystyrene and divinylbenzene (DVB) was designed as a solid support (Figure 2.4A). This type of resin has been improved and became the most common class used for SPPS and well known as gelatinous solid support. A cross-linking of 1-2% of the DVB on the polystyrene beads encourages insolvency, mechanical resistance and solvation of the resin. The solvation of gelatinous resins strongly affects the reactivity of the bead sites.
These supports swell in most solvents and perform as gels, which freely permeated by liquid media enhancing an access to the inner reaction sites of the beads. Solid supports mainly used in SPPS for Boc-SPPS and Fmoc-SPPS are exemplified in Figure 2.4 and Figure 2.5, respectively.

Figure 2.4 The examples of polystyrene bead resins functionalized with chloromethyl group and amine group in Merrifield resin (A) and MBHA resins (B) when the first amino acid of peptide is attached. Both resins are commonly used in Boc-SPPS and the MBHA resin is employed for dendrimer synthesis using Boc strategy in this work.

The solid supports used for lysine-based dendrimer synthesis in this work are MBHA resin and Rink amide MBHA resin in Boc and Fmoc methodologies, respectively. The 4-methylbenzhydralamine (MBHA) resin is a commercial available resin which is also widely used for Boc-SPPS (Matsueda & Stewart 1981). It is a polystyrene bead cross-linked with 1% DVB which is further conjugated to a 4-methylphenylaminomethyl group. The cleavage of the carboxamide (C-terminal amide) from this resin can be achieved by treatment with liquid hydrogen fluoride (HF) or trifluoromethanesulfonic acid (TFMSA). In Fmoc-SPPS, 4-(2’,4’-Dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA (Rink amide MBHA) resin (Bematowicz et al. 1989), a commercially available resin, is commonly used apart from Wang resin, which is a very first developed resin for Fmoc methodology. As it is modified from MBHA, the polymer matrix is therefore the copolymer polystyrene beads cross-linked with 1% DVB before an attachment of Rink amide linker. The Rink amide linker is also modified by Fmoc protected (Novabiochem, Merck Biociences
Ltd. 2005). A modification with alkyl or alkoxy-substituted benzhydrylamines or benzylamines (and incorporation of an acetic acid spacer) permits attachment to amine support via carboxyl group and cleavage of amide products can be accomplished under mild acid condition. Thus this is compatible with the standard 95% TFA cleavage reaction.

![Figure 2.5](image)

**Figure 2.5** Another two examples of polystyrene bead resins generally exploited for Fmoc-SPPS: A is the Wang resin forming an ester linkage after the first amino acid of peptide is attached. B is the Rink amide MBHA resin; the amide linkage can directly be conducted when the first amino acid is attached. The later resin is used for dendrimer synthesis using Fmoc strategy in comparison to Boc strategy.

### 2.2.1.2 Protection and deprotection

An α-amino protecting groups and side chain protection groups of an amino acid have to be compatible with the selected resin described previously. Two types of α-amino protecting groups are mainly utilised in SPPS and withstand the tests of time to become the standards methods in SPPS: an acid-labile tert-butoxycarbonyl (Boc) group and a based-labile 9-fluorenylmethoxycarbonyl (Fmoc) group. The two double-protection strategies are the most practical procedures. In Boc methodology, Boc and benzyl (Bzl) act as an α-amino protecting group (temporary protecting group) and side chain protecting group (permanent protecting group), respectively (Anderson & McGreror 1957). Cleavage of Boc protection group is accomplished by trifluoroacetic acid (TFA), usually 25-50% (v/v) in dichloromethane, or by Lewis acid in polar solvents whereas the final cleavage of the peptidyl resin and side chain
protected (Bzl) requires strong acid includes HF and TFMSA. For Fmoc methodology (Figure 2.7), Fmoc and tert-butyl (t-Bu) (Carpino & Han 1970) behave, respectively, as temporary protecting groups and permanent protecting group; the deprotection of the Fmoc, however, can be achieved under mild basic condition such as dilute liquid ammonia, ethanolamine and piperidine, usually 20-50% in N, N-dimethylformamide (DMF), while the t-Bu only requires high percentages of TFA (95%) for final cleavage. In fact some of Boc-compatible protecting groups can be used in combination with Fmoc chemistry as can be seen in Figure 2.7.

Although Boc-SPPS provides high overall yields and clean peptides (Lebl et al. 1999), not all peptide sequences are entirely stable to such strong acid. Hence, side reactions have been found (Chan & White 2000). The repetitive TFA acidolysis of Boc deprotecting group can lead to an alteration of sensitive peptide bonds as well as acid catalysed side reactions (Smith 1992). HF is a hazardous reagent requiring special equipment for its safe handling, which is expensive and may not be broadly available. These concerns provide an impetus for the development of milder methods. A base-labile condition was earlier demonstrated in a trial assembly of the Merrifield-Dorman tetrapeptide and decapeptide that basic reagents could be used to cleave peptides resin-bound through a base-labile p-carboxybenzyl alcohol linkage (Atherton et al. 1975; Atherton et al. 1978). Importantly, an assignment of base-labile amino protecting group permitted the use of t-butyl or other acid-labile groups as a side chain and C-terminal protection (Chang & Meienhofer 1978). Consequently, the use of HF could be completely eradicated. A wild range of base-labile N-terminal protecting groups have been examined for SPPS (Atherton & Sheppard 1989). An Fmoc protecting group introduced by Carpino and Han (1970) was later proved to be an exceptionally suitable for SPPS (Carpino & Han 1972) and became an efficient alternative to Merrifield technique.
Figure 2.6  Schematic diagram represents protection in the Boc-benzyl strategy (Kate & Albericio 2000). PAM is phenylacetamidomethyl linker.

Figure 2.7  Schematic diagram represents triply orthogonal protection in the Fmoc-t-butyl strategy (Kate & Albericio 2000). BAL is backbone amide linker.
2.2.1.3 Activation and coupling

Extraordinarily high yields together with minor side reactions for the repetitive amide bond formation reactions or peptide couplings are a prerequisite for the achievement of stepwise SPPS. This requires appropriate activation of C\(^\ominus\) carboxylic acid function before a displacement of the activating group by the incoming N\(^\ominus\) amino nucleophile.

Two major categories of activation have been employed: preactivation and \textit{in situ} activation (Kate and Albericio 2000). The former category involves an initial activation of the C\(^\ominus\) carboxylic acid of a protected amino acid to give a stable derivative prior to reaction with an amine in the next step. In the latter category widely exploited in SPPS, an amide bond is formed \textit{in situ} while an amine, carboxylic acid, and condensing agent are mixed together simultaneously. Ideally, the coupling reagents should selectively activate C\(^\ominus\) carboxylic acid of a protected amino acid and remain inert toward the amine.

To date a variety of the coupling reagents have been invented and in recent reports they have been classified into eight groups according to their chemical structures: 1) carbodiimide, 2) imidazolium, 3) phosphonium, 4) uranium, 5) immonium, 6) organophosphorous, 7) acid halogenating and 8) miscellaneous (Han & Kim 2004). The classical prototype of coupling reagent is N,N'-dicyclohexylcarbodiimide (DCC); it was first introduced in 1955 by Sheehan and Hess and remains the most common coupling reagent using in SPPS as its simplicity, cheap price and its particular suitability for the apolar environment of polystyrene-based solid supports. The reaction involving DCC (Sheehan & Hess 1955) and its derivatives efficiently generated O-acylisourea and symmetrical anhydrides under a variety of conditions depicted in Figure 2.8. This leads to the creation of side reactions resulting in the formation of 5(4H-oxazolones) and N-acylureas. These side reactions may be circumvented by using an auxiliary nucleophile such as 1-hydroxybenzotriazole (HOBT, Hudson 1988) and 1-hydroxy-7-azabenzotriazole (HOAT, Carpino 1993) as an auxillary coupling reactant, which counter the O-acylisourea and turn it into a less reactive form yet still relatively effective acylating agent (Figure 2.8). The reaction rate is, therefore, enhanced as well as the reduction of the racemisation.
Figure 2.8  Coupling reactions of amino acids using dicyclocarbodiimide (DCC) as coupling reagent in combination with HOBT result in the production of reactive intermediate including O-acylisourea, symmetric anhydrides and the HOBT ester of protected amino acid.

Other coupling reagents were soon proposed after the discovery of the HOBT as racemisation suppressant in the presence of carbodiimide coupling reagents (König & Geiger 1970) includes benzotriazol-1-ylxytris(dimethyl-amino)phosphonium hexafluorophosphate (BOP, Castro et al. 1975), O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, Dourtoglou & Gross 1984;Knorr et al. 1989), benzotriazol-1-ylxytripyrrrolidino)-phosphonium hexafluorophosphate (PyBOP®, Coste et al. 1990;Høege-Jensen et al. 1991), O-benzotriazol-1-yl-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU, Gausepohl et al. 1991) and bromotri(pyrrolidino)- phosphonium hexafluorophosphate (PyBrOP®, Coste et al. 1990;Frérot et al. 1991) as can bee seen in Figure 2.9. These new coming reagents
possess an additional advantage because they acquire the effective additives as a leaving group which is then released upon reaction of the phosphonium or ammonium salt with a carboxylate of an amino acid to form an active intermediate. This active intermediate is stable in the presence of a protected amino acid and selectively reacts to the corresponding carboxylate. Therefore, the use of base is required with these reagents.

Figure 2.9 Chemical structures of coupling reagents commonly used with carbodiimide in SPPS to circumvent side reactions and suppress racemisation (Novabiochem, Merck Biociences Ltd. 2005).

In this work HBTU was selected as the coupling reagent in combination of a tertiary amine base, diisopropylethlamine (DIEA or DIPEA) to ensure that the carboxylated group of the $N\varepsilon$-amino protected amino acid is remnant in its anionic form. Thus its reaction with the uronium cation can be accomplished and subsequently generates a highly reactive acycloxyuronium intermediate, as illustrates in Figure 2.10. HOBT is also selected as an auxiliary coupling reagent with HBTU and DIEA to reduce side reactions and increase reaction rate. As the acycloxyuronium intermediate can then be readily transformed into the reactive esters of HOBt and the symmetrical anhydride likewise the case of carbodiimides. These reactive esters behave as acylating agents to the nucleophilic amine of the peptide chain (Jones 1999).
Figure 2.10 Coupling reactions of amino acid involved by HBTU coupling reagent: the reactive intermediate of acyloxyuronium is created after the first coupling between the anionic carboxylate group and HBTU. This intermediate then reacts to HOBT forming HOBt ester, which later forms peptide bond with second amino acid. The intermediate of the symmetrical anhydride are also created and can readily form a peptide bond with the second amino acid. Some of the symmetrical anhydride intermediates react to HOBT transforming to HOBt ester before amide bond formation.

2.2.1.4 An assessment of complete coupling reaction (Ninhydrin test)

In SPPS the terminal amino groups should be completely coupled with the amino acid derivatives to avoid construction of incomplete peptide chains. The coupling reaction must be repeated if the terminal free amino groups remain. Therefore, the determination of unreacted terminal amino groups is a crucial step to control peptides synthesis. The most common method employed for qualitatively monitoring either the presence or absence of these free amino groups during deprotection or coupling process is based on the reaction of ninhydrin reagents with small amounts of the resin in the well known “ninhydrin test” or “Kaiser test” (Kaiser et al. 1970). This colorimetric detection is simple, rapid and sensitive enabling an indication whether greater than 99% of the terminal amino groups are reacted.
Three reagents were prepared for this test: 1) 5 g of ninhydrin in 100 ml ethanol, 2) 80 g liquified phenol in 20 ml ethanol and 3) 2 ml a 0.001 M aqueous solution of potassium cyanide in 98 ml of pyridine. The test can be done by taking a sample of a few resin beads and washing the resin several times with ethanol, transferring the resin beads into a small glass test tube and adding 2 drops of each reagent. The sample is mixed well and heated at 120 °C for 5 min with occasionally swirling. The remaining white colour of bead with clear to yellow solution (negative test) indicates a complete coupling reaction. A dark blue colour developed on beads and in the solution (positive test) illustrates the deprotection of terminal amino acids (during deprotection) or incomplete coupling reaction (during coupling reaction).

2.2.1.5 Cleavage of the synthesized dendrimer from the solid support

The final process of peptide synthesis involves the detachment of the peptide from the resin simultaneously with the removing of all the side chain protecting groups of the amino residues. The proper preparation of peptide prior to cleavage is essential in preventing side reactions and incomplete cleavage and deprotection of side chain protection. All peptide resin should be properly washed and dried. The cleavage method is depending on the type of resin used and choice of side-chain protecting groups.

2.2.1.5.1 Boc resin cleavage

For Boc-based peptide synthesis, the cleavage of peptides and side chain deprotection necessitate strong acid such as HF and TFMSA. HF is the most versatile and broadly utilized because it possesses many desired characteristics for side chain deprotection in peptide synthesis (Sakakibara 1971). Nevertheless, HF is a tremendously toxic, hazardous, corrosive, and volatile liquid; all the performance involves HF must be strictly conducted in fulfillment with standard safety regulations. An excellent description of properties of HF, its safety data sheet and technique for handling HF in SPPS can be read elsewhere (Sakakibora 1971).
Two general methods are regularly used for Boc resin cleavage: standard HF cleavage and low-high HF cleavage. In the first method the cleavage is usually carried out at 0-5 °C for 30-60 min. To minimize the possible side reaction rising during the cleavage and deprotection processes, controlling both temperature and duration of reaction are the most concerned. Scavengers play an important role in lowering the possibility of side reactions. These side reactions emerge during the deprotection process and render highly reactive carbonium ion through an S_N1 mechanism (Tam et al. 1983). The scavengers are, therefore, needed, and one of the most common used is anisole. The low-high procedure reported by Tam et al., (1983), using low concentrations of HF in a large amount of scavenger. The mechanism of reaction is transformed from the normal S_N1 to S_N2 which does not engage carbonium ion production. The major downsides of this method are the long cleavage time, the large amount used of DMS and the offensive odors from the formation of thiol scavenger adduct.

2.2.1.5.2 Fmoc resin cleavage

Cleavage peptide from Fmoc-based resin is simply achieved by using a high concentration of TFA. However, care must be taken because the TFA is an extremely corrosive liquid. The cleavage condition generally used is 1 g of resin per 10-25 ml of 95% TFA: 2.5% water: 2.5% scavengers (triisopropylsilane, TIS). Scavengers react with the linker of the resin preventing side reactions (Fields & Noble 1990). A small amount of the peptide resin is recommended for optimization the cleavage condition as the reaction time is depending on amino acids containing in peptides, types of resins and scavengers. The reaction should be carried out at room temperature with occasional stirring.

In this present work the dendrimer is cleaved from resin using standard HF procedure and 95% TFA in for Boc and Fmoc strategies, respectively. The peptide dendrimer is subsequently precipitated by cold anhydrous ether to eradicate scavengers and separated by filtration. The peptide is then extracted by suitable concentrations of glacial acetic acid or TFA before lyophilisation. The compound is re-suspended in distilled water and lyophilized to remove all acid residual. The product is kept in dry conditions at -20 °C until used.
2.3 Experimental

2.3.1 Materials

4-Methylbenzhydrylamine (MBHA) resin, Rink Amide MBHA resin, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), N-hydroxybenzotriazole (HOBt), the protected Boc-Gly-OH, Boc-Lys(Boc)-OH (DCHA salt), Fmoc-Gly-OH and Fmoc-Lys(Fmoc)-OH were purchased from Novabiochem, UK. Diisopropylethylamine (DIEA), Magnesium sulphate dried, Potassium hydroxide pellets, Sulphuric acid, Glacial acetic acid and Ethyl acetate were purchased from BDH, UK. Dimethylformamide (DMF) was purchased from Rathburn, UK. Trifluoroacetic acid (TFA) was obtained from Halocarbon Product Corporation, USA. Hydrogen fluoride gas (HF) was obtained from BOC, UK. Pleated dialysis tubing (Snakeskin™), 3500 (MWCO), was purchased from Pierce Chemical Company, USA. Dichloromethane (DCM), ethanol, methanol and poly-L-lysine hydrobromide (Mw 9,600, 23,800 and 121,000 Da) were purchased from Sigma-Aldrich, USA.

2.3.2 Methods

2.3.2.1 Activation of Boc-Lys(OH)-Boc-DCHA salt

In brief, the protected amino acid, Boc-Lys(Boc)-OH.DCHA, 1.4 g (2.68 mmol) was first suspended in 10.4 ml ethyl acetate in a separating funnel. Next 3.12 ml (3.2 mmol) of ice-cold 2 M H$_2$SO$_4$ was added into the funnel and the mixture was shaken until all dissolved. The top layer of ethyl acetate was removed and kept aside. The aqueous layer (bottom layer) was diluted with 10 ml of cold water and extracted with 10 ml of ethyl acetate (x2), combining all the ethyl acetate layers and then washing with 10 ml of water (x2) prior to drying with MgSO$_4$. The ethyl acetate part was filtered and the ethyl acetate was removed in a rotary evaporator at the temperature less than 40 °C. Complete solvent removal could be achieved under high vacuum in a desiccator over fresh NaOH.
2.3.2.2 Coupling of dendrimer synthesis

The poly-lysine based dendrimers (Gly-Lys<sub>63</sub>(NH<sub>2</sub>)<sub>64</sub>) were synthesized using stepwise SPPS by Boc and Fmoc methodologies using MBHA (1g, substitution 0.67 mmol/g) and Rink amide MBHA resins (1g, substitution 0.78 mmol/g) respectively (Figure 2.11). The detail of dendrimer synthesis using Boc methodology can be found elsewhere (Al-Jamal et al. 2006). Briefly, the resin was first allowed to swell in DMF (three times volume of resin) at room temperature in the reaction vessel and never allowed to dry through whole process of peptide couplings. Boc-Gly-OH (4 molar equivalents relative to resin loading (1 equiv)), HBTU (4 equiv), HOBt (4 equiv) were dissolved in minimum amount of DMF. DIEA (8 equiv) was finally added to the solution and the solution was then added into the vessel to make the first coupling. The completion of coupling reaction was checked by the ninhydrin test. When the reaction was complete, the second coupling was performed. N-terminal deprotection was carried out with 100% TFA for 1 min (x2). Another six successive couplings/deprotections were carried out with four-fold molar equivalent of activated Boc-Lys(Boc)-OH.DCHA (4,8,16,32,64,128 equiv), HBTU (4,8,16,32,64,128 equiv), HOBt (4,8,16,32,64,128 equiv) and DIEA (8,16,32,64,128,256 equiv) as illustrated in Figure 2.8. However, the swelling of the resin was reduced after the 3<sup>rd</sup> coupling, hence DMF was replaced by a mixture of DMF/NMP (1:1 ratio) to achieve better resin swelling and more efficient coupling. Then the resin was carefully washed with DCM (x2) and dried under vacuum. The compound was cleaved from the resin by standard HF method (method A in Appendix I). After cleavage, the dendrimer was precipitated with ether to remove fluoride residues. The precipitated dendrimer and resin were filtered, and redissolved with 20% glacial acetic acid or 0.1% TFA prior to lyophilisation. The lyophilized material was further dried over P<sub>2</sub>O<sub>5</sub> and stored over silica gel.

A similar concept was applied in Fmoc methodology, yet the peptides were assembled on Rink amide MBHA resin (1g, substitution 0.78 mmol/g) using Fmoc-Gly-OH, Fmoc-Lys(Fmoc)-OH and HOBt (4 molar equivalents relative to resin loading (equiv)) and DIEA (8 equiv). The deprotection of amino acid was accomplished by
20% piperidine in DMF. The dendrimer was detached from the resin using the standard TFA cleavage method (method B in Appendix I).

2.3.2.3 Mass Spectrometry (MS)

Mass spectrometry (MS) particularly electrospray ionization (ESI) MS and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS has found widespread utility for peptide characterization. In principle, there are three main steps involved in MS: compound vaporization, ionization of those neutral molecules in their vapour phase and separation the ions in accordance with their mass-to-charge ratios (m/z). As a result, the molecular weight information of the peptides can be directly verified.

The dendrimers synthesized by both Boc and Fmoc method were analysed using MALDI-TOF MS: the compounds (collected after cleavage and ether precipitation) were run on a MALDI-TOF mass spectroscopy (Applied Biosystems Voyager System 6286 De-Pro). The sample solution, approximately $10^{-10}$M of the dendrimer (10 μl) in 1:1 ratio of acetonitrile: 0.1% TFA was mixed with saturated matrix, sinapinic acid prior to be placed on the sample plate. Sample volume per one run was 2 μl. The mixture was then leave to dry at room temperature and the plate was inserted into the machine. The molecular weight of the sample was analysed and the result was recorded as a mass spectrum.

2.3.2.4 Nuclear magnetic resonance (NMR)

NMR is a spectroscopic technique which involves the measurement of the absorbance of radiofrequency radiation occurring when the certain types of atomic nuclei, such as $^1$H, $^{13}$C and $^{15}$N, are located under strong magnetic fields. This radiation is exquisitely sensitive to the surrounding of the nuclei and hence allows chemical structures of the molecules to be determined. NMR has been the most powerful method for dendrimer characterization (Caminade et al. 2005).
Figure 2.11 A schematic diagram demonstrates the overview of lysine-dendrimer (Gly-Lys₆₃(NH₂)₆₄) synthesis using Boc/Fmoc strategy.
The dendrimer was characterised using a one-directional proton NMR (£H-NMR) based on the method explained by Zloh et al (2005). The sample, 6-10 mg, was dissolved in D$_2$O and placed into a clean NMR tube. The sample was run by a Bruker ADVANCE 500 MHz MNR instrument at 300 K with 30° pulse excitation angle and relaxation decay of 25. A £H-NMR spectrum was acquired with 32768 data points and analysed under Topspin version 1.3 NMR processing software with or without water suppression.

2.3.2.5 High performance liquid chromatography (HPLC)

A reverse phase-HPLC (RP-HPLC) is the most frequently routine method for characterization and purification of synthesis peptide and peptide dendrimers. The solid support inside the RP-HPLC column is usually bound with the alkyl groups usually containing 4, 8 or 18 carbons; the longer the alkyl length the more efficient the separation will be. Previously the difficulty in separation and characterisation this type of lysine-based dendrimer using Vydac C$_4$ column RP-HPLC has been reported by Al-Jamal, K.T., (2006).

In this work the dendrimer was therefore characterised and purified using Vydac C$_8$-column with RP-HPLC Hewlett Packard. Analytical separations were carried out using Vydac RP-C4 (5µm, 4.6mm x 250 mm) and Vydac RP-C8 columns (5µm, 4.6mm x 15mm). Preparative separations were carried out using Vydac RP-C8 column (10µm, 22mm x 15mm). The dendrimer was eluted with a gradient system consisted of 0.1% (v/v) TFA in water (solution A) and 100% (v/v) Methanol (solution B) which achieved by two microprocessors-controlled Gilson 302 single piston pumps. The condition of separation was 90% of solution A and 10% of solution B for 5 min then 5`: 95% (solution A: solution B) for 5 min following by 90%:10% (solution A: solution B) for 5 min. All the solvents used in the experiment were HPLC grade. Separation was monitored by Water 486 tunable detector at 230 nm the data was analysed using PC/Chrom software.
2.3.3 Results and discussion

Cationic poly-lysine-based dendrimer, Gly-Lys$_{63}$(NH$_2$)$_{64}$, was acquired by both Boc (compound 1a) and Fmoc (compound 1b) solid phase peptide synthesis with details as the following.

**Compound 1a**

Yield: 64.10%

C$_{380}$H$_{762}$O$_{64}$N$_{128}$ (8149.05) m/z (%) 8149 (100) 8136(92) 9021 (98) 6952(68) †

$^1$H-NMR (D$_2$O) δ 3.84-4.35 (t, 65H, α-CH), 2.95-3.23 (t, 126H, ε-CH), 1.25-1.90 (m, 378H, β, γ, δ-CH)

**Compound 1b**

Yield: 30.04%

C$_{380}$H$_{762}$O$_{64}$N$_{128}$ (8149.05) m/z (%) 8204 (100) 8308 (25) †

$^1$H-NMR (D$_2$O) δ 3.84-4.35 (t, 65H, α-CH), 2.95-3.23 (t, 126H, ε-CH), 1.25-1.90 (m, 378H, β, γ, δ-CH) ‡

† Although an envelope was observed as the sample was not purified, the peak for the expected molecular weight is observed.

‡ The coupling was not observed instead broad peaks were acquired

2.3.3.1 MS

Mass spectra of the dendrimer, (Gly)(Lys)$_{63}$(NH$_2$)$_{64}$, synthesized using Boc (compound 1a) and Fmoc SPPS (compound 1b) are illustrates in Figure 2.12. As the compounds were not purified by HPLC, the higher or the lower generation of the desired dendrimer and the impurity could be found in both methodologies. However, after the purification the expected molecular weight of the dendrimer could not be indicated (data not show). The reason for this could not be explained; the purified compounds were, therefore, characterized further by NMR and HPLC.
Figure 2.12  Mass spectrum of \((\text{Gly})_6(\text{Lys})_6(\text{NH}_2)_4\) dendrimer synthesised by Boc (A=compound 1a) and Fmoc (B=compound 1b) strategy: compounds were dissolved in 1:1 ratio of acetonitrile: 0.1% TFA and mixed with saturated matrix, sinapinic acid prior to be analysed by MADI-TOF MS. The noise signals were observed as the compounds were purified by HPLC.
2.3.3.2 NMR

The characterisation and the supramolecular assemblies of the cationic lysine-based dendrimer in solution were previously studied using $^1$H NMR (Zloh et al. 2005). The lysine unit of the dendrimer could be classified into two main categories: interior lysine (Lys1, Lys2a and Lys2e in blue area) and exterior lysine (Lys3aa, Lys3ae, Lys3ee and Lys3ea) as illustrated in Figure 2.13. This also categorised the lysine residues according to their environment as interior and exterior residues. The difference between them is that the interior lysine containing amide protons (green circle) due to the peptide bond formation whereas the exterior lysine (terminal lysine) containing amine protons (pink circle).

Figure 2.13 Assignment of the internal and external lysines and lysine residues inside structure of partially the 3rd generation lysine-based dendrimer, $(\text{Lys})_7(\text{NH}_2)_k$: R represents dendrimer core (Glycine). The figure was modified from Zloh et al. 2005. The blue area allocates internal lysines and the external lysines locate outside the blue area. The green and pink spheres indicate the amide protons and amine protons, respectively.
The $^1$H NMR spectra of Gly-Lys$_{63}$(NH$_2$)$_{64}$ dendrimer synthesised by Boc (compound 1a) and Fmoc strategy (compound 1b) are demonstrated in Figure 2.14 and 2.15, respectively. Since this compound had once synthesized and characterised by the similar method (Al-Jamal et al. 2006), the interpretation of the compound 1a and 1b was then compared with that reference (Table 2.1). The first three peaks upfield of the interior amide (internal H$_{oc}$), external amide (external H$_{oc}$), interior amide (internal H$_e$) and exterior amine (external H$_e$) could be easily found in both spectra. The H$_{oc}$ and H$_e$ of the interior lysine residues were found shifted downfield while that of the exterior lysine residues shifted upfield.

**Figure 2.14** $^1$H-MNR spectrum of Gly-Lys$_{63}$(NH$_2$)$_{64}$ synthesised by Boc strategy in D$_2$O
Figure 2.15 $^1$H-MNR spectrum of Gly-Lys$_6$\textsubscript{(NH$_2$)$_6$} synthesised by Fmoc strategy in D$_2$O

Table 2.1 The assignment of $^1$H NMR spectra of the compound 1a and 1b in comparison to that of a reference compound

<table>
<thead>
<tr>
<th>Protons</th>
<th>Chemical shift of compound 1a</th>
<th>Chemical shift of compound 1b</th>
<th>Reference (Al-Jamal et al. 2006)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal H$_a$</td>
<td>4.213 (2.16)</td>
<td>4.233 (3.35)</td>
<td>4.521-4.532 (2.28)</td>
</tr>
<tr>
<td>Assignment (area)</td>
<td></td>
<td></td>
<td>4.422-4.436</td>
</tr>
<tr>
<td>External H$_a$</td>
<td>3.945 (2.03)</td>
<td>3.930 (0.31)</td>
<td>4.173 (2.21)</td>
</tr>
<tr>
<td></td>
<td>3.903</td>
<td>3.900</td>
<td>4.090-4.117</td>
</tr>
<tr>
<td>Internal H$_e$</td>
<td>3.203-3.217 (6.58)</td>
<td>3.144-3.228 (7.02)</td>
<td>3.365-3.451 (5.27)</td>
</tr>
<tr>
<td>External H$_e$</td>
<td>2.986-3.026 (6.57)</td>
<td>2.990-3.037 (2.97)</td>
<td>3.190-3.234 (4.36)</td>
</tr>
<tr>
<td>External H$_g$, Internal H$_p$, External H$_6$</td>
<td>1.665-1.729 (20.38)</td>
<td>1.673-1.765 (13.07)</td>
<td>1.941-2.096 (14.10)</td>
</tr>
<tr>
<td>Internal H$_p$, External H$_t$, Internal H$_t$</td>
<td>1.401-1.533 (23.56)</td>
<td>1.401-1.534 (17.51)</td>
<td>1.548-1.752 (16.33)</td>
</tr>
</tbody>
</table>
In contrary, the interior of $H_\beta$, $H_7$, $H_8$ proton shifted upfield whereas that of the exterior proton shifted downfield. A similar trend was found in both compounds 1a and 1b and in good agreement with the reference compound. Although there was a slight shift of the spectrum to the left (Table 2.1), the area of the peak remain overlap to that of the reference compound. We assume that this might be due to the difference in the conditions of use for purification. HPLC may create a change in conformation of the dendrimer molecules. As both strategies provided the similar assigned spectra, the method of synthesis was not the issue.

2.3.3.3 HPLC

There was a recent reported difficulty in the separation and purification of the Gly-Lys$_{63}$(NH$_2$)$_{64}$ dendrimer as it provide short retention time and could not be separated from the smaller generation and its impurity (Al-Jamal, et al. 2006). A similar problem was found in both compounds as can be seen in Figure 2.16. It was envisaged that the peaks (p) of impurity (p1-p5) could not be separated from the peak of the desired dendrimer (p6). The rest of the peaks obtained from the solvent B in gradient system (MeOH) as shown in the insert in Figure 2.16. The percentage of the impurity found in the compound synthesised by Fmoc SPPS was higher than that was found in the compound synthesis by Boc SPPS (table in Figure 2.16 and 2.17). The chromatogram of the dendrimer synthesis by Boc methodology was illustrates in Figure 2.17. Only 7.18 % of the impurity was found in compound synthesized using Boc SPPS. Although the retention time of the desired dendrimer (p4 in Figure 2.17) was found after 5 min of injection in $C_8$ column, the dendrimer could be separated from its contaminants. This may be due to the longer of the alkyl chain with bound to the solid support of HPLC column resulted in the higher hydrophobicity of the stationary phase which capable of retaining the dendrimer and liberating the impurity (p1-p3 and p6). Since, solvent B in gradient system (MeOH) involved in the creation of peak number 5 and 7 (the insert picture in Figure 2.17), the desired dendrimer could, therefore, be separated by this system. The purification of the dendrimer synthesized by both methodologies was purified using this condition in $C_8$ RP-HPLC.
<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention time (min)</th>
<th>Area</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.337</td>
<td>1.429</td>
<td>2.93</td>
</tr>
<tr>
<td>2</td>
<td>3.908</td>
<td>2.943</td>
<td>6.04</td>
</tr>
<tr>
<td>3</td>
<td>4.367</td>
<td>5.844</td>
<td>12.00</td>
</tr>
<tr>
<td>4</td>
<td>4.950</td>
<td>3.810</td>
<td>7.82</td>
</tr>
<tr>
<td>5</td>
<td>5.646</td>
<td>4.526</td>
<td>9.29</td>
</tr>
<tr>
<td>6</td>
<td>6.737</td>
<td>20.825</td>
<td>42.74</td>
</tr>
<tr>
<td>7</td>
<td>8.183</td>
<td>3.163</td>
<td>6.49</td>
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<tr>
<td>8</td>
<td>9.063</td>
<td>0.929</td>
<td>1.91</td>
</tr>
<tr>
<td>9</td>
<td>9.575</td>
<td>0.750</td>
<td>1.54</td>
</tr>
</tbody>
</table>

Figure 2.16  RP-HPLC chromatograms of Gly-Lys₆₃(NH₂)₆₄ synthesised by Fmoc strategy obtained under the gradient condition: 90% of solution A (0.1% TFA) and 10% of solution B (MeOH) for first 5 min, 5%: 95% (solution A: solution B) for the next 5 min and 90%:10% (solution A: solution B) for 5 min at constant flow rate of 1ml/min detected at 230nm in C₄ column RP-HPLC. The inserted picture is a chromatogram of 10% MeOH in the same gradient condition.
Figure 2.17  RP-HPLC chromatogram of Gly-Lys$_{60}$(NH$_2$)$_{64}$ synthesised by Boc strategy obtained under the gradient condition: 90% of solution A (0.1% TFA) and 10% of solution B (MeOH) for first 5 min, 5%: 95% (solution A: solution B) for the next 5 min and 90%:10% (solution A: solution B) for 5 min at constant flow rate of 1ml/min detected at 230nm in C$_8$ column RP-HPLC. The insert picture is chromatogram of 10% MeOH in the same gradient condition.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention time (min)</th>
<th>Area</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.496</td>
<td>1.201</td>
<td>0.39</td>
</tr>
<tr>
<td>2</td>
<td>2.962</td>
<td>2.993</td>
<td>0.96</td>
</tr>
<tr>
<td>3</td>
<td>3.396</td>
<td>1.311</td>
<td>0.42</td>
</tr>
<tr>
<td>4</td>
<td>5.429</td>
<td>289.356</td>
<td>92.82</td>
</tr>
<tr>
<td>5</td>
<td>5.963</td>
<td>3.573</td>
<td>1.15</td>
</tr>
<tr>
<td>6</td>
<td>7.838</td>
<td>1.965</td>
<td>0.63</td>
</tr>
<tr>
<td>7</td>
<td>12.817</td>
<td>11.334</td>
<td>3.64</td>
</tr>
</tbody>
</table>
Summary

Two techniques were used to understand the diffusion phenomena of nanocarrier systems: dynamic light scattering (DLS) and fluorescent recovery after photobleaching (FRAP). DLS was employed to measure the diffusion coefficient of positively (108±7 and 495±23 nm) and negatively charged latex nanospheres (48±7, 91±9.8 and 483±10 nm) in different types of media, water, aqueous glycerol solutions and hydroxypropyl methylcellulose (HPMC) gels. The translational diffusion coefficients \(D\) of these latex spheres in water were found to be 13.00 (±0.12), 5.11 (±0.06), 0.89 (±0.01) \(\mu\text{m}^2/\text{s}\) for 48, 91 and 483 nm carboxyl latex nanospheres (CLN), and 3.26 (±0.01) and 0.88 (±0.03) \(\mu\text{m}^2/\text{s}\) for 108 and 495 nm amino latex nanospheres (ALN), respectively. In Newtonian aqueous glycerol solutions, as anticipated, the diffusion could be predicted by the Stokes-Einstein relationship over a range of system viscosities. In HPMC gels the results showed the deviation of the diffusion coefficient from the Stokes-Einstein equation when the bulk viscosity of the medium was increased. From the \(D\) values, the “effective” or “microscopic” viscosities of the HPMC medium were calculated, and ranged from 0.899 - 0.925 mPa.s. Because of some limitations of DLS, the FRAP technique could overcome such difficulties and was successfully used to determine the diffusion coefficient of dendrimers in aqueous solution and actin gels. The diffusion coefficient in an actin gel of an intrinsically fluorescent poly-lysine dendrimer with 6.5 nm in diameter, \((\text{Gly})(\text{Lys})_{63}(\text{NH}_2)_{64}\), measured by the FRAP method, was found to be 23.75± 3.22 \(\mu\text{m}^2/\text{s}\)
3.1 Introduction

The work in this chapter was to ensure that the techniques used to measure the diffusion of our nanocarrier systems including the nanospheres and the dendrimer in particular types of media mimicking cellular entities were appropriate and accurate. Hence, experiments were first aimed to investigate the diffusion phenomenon of the nanoparticles (in particular nanospheres and the dendrimer) in simple media such as pure solvent (water) and dilute polymer solutions. The second aim of this work was to use more complex media, e.g. crosslinked of HPMC gels, and biological elements (actin gel) as models to understand the diffusion phenomenon of the nanocarriers inside cells. The principles of diffusion together with discussion of the diffusion of particles in gels are addressed here.

The diffusion of particles, molecules and ions is a phenomenon encountered in nature and it can be scrutinized in micro- and nanoscopic scales. Likewise thermal, electrical and fluid flow emerges as a transfer from high (temperature/electrical potential) level to low (temperature/electrical potential) level until the dissimilarity no longer exists. Diffusion is originated by the random motion of an individual particle or molecule associated with a concentration gradient to accomplish equilibrium (Clussler 1997). The duration of the diffusion process is dependent on the nature of the diffusing system and the diffusion rate of molecules, for instance, in a gas is found to be faster than that in liquid and solid, respectively. This is also true for nanoparticles. The diffusion and transport of nanoparticles in simple and complex media are important elements in understanding their behaviours as drug carriers and targeting agents. Using photon correlation spectroscopy (PCS), the translational diffusion coefficient \(D\) of nanoparticles can be directly measured from the decay rate of the time autocorrelation function (Pecora 2000) and transformed into a hydrodynamic diameter using the Stokes-Einstein equation.

Many studies of the diffusion of particles and macromolecules have been made in aqueous isotropic and Newtonian systems (Masaro & Zhu 1999). Understanding the movement of nanoparticles in viscous and complex media is a prelude to understanding the diffusion and translocation of nanoparticles inside cells and tissues.
Several attempts have been made to increase the viscosity of the continuous phase of particle dispersions to imitate the viscosity of cellular contents (Masaro & Zhu 1999; Bremmell et al. 2001; Westrin & Axelsson 1991). We have carried out diffusion experiments with negatively and positively charged polystyrene latex nanospheres in aqueous glycerol solution and HPMC gels using PCS. In gels the bulk viscosity does not, as is well known, reflect the viscosity of the medium in which the particles move. This is rather the intrinsic viscosity or effective viscosity (Florence et al. 1973; Westrin & Axelsson 1991) which we have estimated here in HPMC gels.

“A gel is a polymer-solvent system containing a three-dimensional network of quite stable bonds which are almost unaffected by thermal motion. If such a polymer network is surrounded by the solvent-the system can be arrived at by swelling of solid polymer or by reduction in the solubility of the polymer in the solution- the system is a gel regardless of whether the network is formed by chemical or physical bonds” (Florence and Attwood, 1998). A gel is formed when the concentration of the polymer in the system reaches a critical concentration of gelation (Florence & Attwood 1998). Gels can be classified based on the types of crosslinking, polymer, shape and size, and types of solvents (Derosi et al. 1991).

Detail of macromolecule as diffusant in gels has been reviewed elsewhere (Lauffer 1961; Crank 1975; Muhr & Blanshard 1982; Westrin & Axelsson 1991). If there is only one solute diffusing inside the gel, the diffusion coefficient ($D_\text{G}$) is independent on the position ($x$) and the concentration ($C_\text{G}$) of diffusant (Westrin & Axelsson, 1991). The diffusion flux $J$ in the x-direction is given by Fick’s first law (Martin 1993):

$$ J = -D_\text{G} \frac{\partial C_\text{G}}{\partial x} \hspace{1cm} (3.1) $$

where $C_\text{G}$ is the amount of solute per unit volume of gel.

In gels, the particles can only diffuse in the liquid void phase and they have to detour around the impermeable obstacles or “solid wall” of the polymers chains. This effectively increases the path length for traveling particles and thus decreases the
coefficient of diffusion. The diffusion coefficient in this case is generally defined as an effective diffusion coefficient \((D_e)\) by

\[ J = -D_e \frac{\partial C_L}{\partial x} \]  

\[(3.2)\]

where \(C_L\) is the amount of solute per unit volume of the liquid void phase within the gel. The relationship between \(C_G\) and \(C_L\) is usually defined as

\[ C_G = \varepsilon C_L \]  

\[(3.3)\]

where \(\varepsilon\) is the void fraction accessible to the diffusing solute. Thereby the diffusion coefficient and the effective diffusion coefficient are related by the equation

\[ D_e = \varepsilon D_G \]  

\[(3.4)\]

Where \(\varepsilon\) is defined as

\[ \varepsilon = 1 - \phi_p \]  

\[(3.5)\]

for the low molecular weight solutes and \(\phi_p\) is the polymer volume fraction of the gel.

There are two main reasons why the effective diffusion coefficient in a gel \((D_e)\) is lower than the corresponding diffusion coefficient in water \((D_o)\). Firstly, the polymer reduces the available diffusion volume to a fraction of the total - the exclusion effect. The second is the obstruction effect - impermeable segments of the polymer molecules increasing the path length for a diffusant. One can define a tortuosity factor \((\tau)\), both \(\varepsilon\) and \(\tau\) being incorporated in equation (3.6):

\[ \frac{D_e}{D_o} = \frac{\varepsilon}{\tau} \]  

\[(3.6)\]

However, it is difficult to determine \(\varepsilon\) and \(\tau\) values by experiment. The polymer volume fraction \((\phi_p)\) has been successfully employed to estimate the effect of polymers in decreasing diffusion coefficients (Mackie & Meares 1955b; Mackie & Meares 1955a) (Eq. 3.7), viz.

\[ \frac{D_G}{D_o} = \frac{(1-\phi_p)^2}{(1+\phi_p)^2} \]  

\[(3.7)\]

By substituting \(D_G\) for \(D_e\) from equation (3.4) and (3.5), equation (3.7) can be written as

\[ \frac{D_e}{D_o} = \frac{(1-\phi_p)^2}{(1+\phi_p)^2} \]  

\[(3.8)\]
and thus the effective diffusion coefficient can be predicted. The diffusion coefficient at infinite dilution, $D$, of a sphere of radius $r$ moving in a continuum of viscosity $\eta$ can be obtained from the Stokes-Einstein equation.

$$D = \frac{kT}{6\pi\eta r} \quad \text{(3.9)}$$

where $k$ is Boltzmann’s constant. Equation (3.9) was derived on the assumption that there is no slip at the surface of the diffusing sphere. Therefore, from equation (3.9), equation (3.8) can be rewritten as

$$\eta_e = \eta_{aq} \frac{(1+\varphi_r)^2}{(1-\varphi_r)^3} \quad \text{(3.10)}$$

Where $\eta_{aq}$ and $\eta_e$ are viscosity of water and effective viscosity of a gel, respectively.

Although the diffusion of the particles can be measured using dynamic light scattering, this method is limited when the solvent contains high concentration of salt or polymer (Yu & Russo 1996). This may cause thermodynamic nonideality such as a temporal aggregation, flocculation and clustering of the particles which compromises the sensitivity and precision of the scattering system. Fluorescence recovery after photobleaching (FRAP) has been established and may be a practical tool to access such problem as it is almost insensitive to the salt concentration (Yu & Russo 1996). FRAP, also called fluorescence photobleaching recovery (FPR) and microphotolysis, exploits the fluorescence technology to studying the mobility characteristics of molecules and particles on a microscopic level (Elson & Qian 1989).

For more than 30 years FRAP has been employed to evaluated the translational diffusion of various types of molecules and particles in a wide range of media, for example aqueous solutions, gels (Ekani-Nkodo & Fygenson 2003), extracellular matrices (articular cartilage (Leddy & Guilak 2003), cystic fibrosis sputum (Braeckmans et al. 2003), tumour (Brown et al. 2004), tissue (Sniekers & van Donkelaar 2005), living cells (Goodwin & Kenworthy 2005; Braga et al. 2004), cell nucleus (Houtsmuller & Vermeulen 2001; Carrero et al. 2003) and cell membrane (Chen et al. 2006). This technique has also been developed for studying the diffusion and intracellular binding within the cells (Wachsmuth et al. 2003). An intensive
review on the FRAP technique and its application can be found in Meyvis, T.K.L, (1999). The applications of FRAP in drug and DNA delivery has recently been established for investigating the dynamic mobility of viral vector (Cui et al. 2005) and for studying the release of active compound from dextran-based hydrogel (Van Tomme et al. 2005).

Since it was first invented in 1957, confocal microscopy has been developed to be applicable to various fluorescent techniques (Shappard 1999) as well as biological techniques. As a result of an advance in confocal scanning laser microscopy (CSLM) a FRAP experiment can be easily performed because a bleaching geometry can be defined in the confocal software. This offers CSLM as a versatile standard method for monitoring dynamic diffusion of molecules and particles in diverse systems. Not only does FRAP require a small volume of sample, but the measurements can also be carried out without the direct passage of the particle or molecules through membranes. In addition, samples that are sensitive to mechanical treatment, for example biological gels and mucus, can preserve their physicochemical properties as well as their biological content during the experiment (Meyvis et al. 1999).

Although FRAP models for non-scanning fluorescent microscopes have been well established, for confocal scanning laser microscope there are few FRAP models. Both two-dimensional (2-D) and three-dimensional models (3-D) have been constructed based on a numerical approach. A practical 3-D model has been developed to be applied to objective lenses of low numerical aperture (NA) on a CLSM which using a stationary laser beam for bleaching and a line-scanning beam for recording the fluorescence recovery. However the practical use of this model on all commercial CSLMs is not possible. A three-dimensional FRAP model with a uniform disk-shaped bleaching arbitrary can be applicable to any modern CSLM equipped with the feature to bleach arbitrary regions. This FRAP model was employed in this work for observing the dynamic mobility of nanoparticles.

The diffusion coefficient of the fluorescent molecules can be measured in three steps. First, the bleaching geometry is defined inside the sample using confocal software.
Second, the molecules inside the defined geometry are bleached with a high attenuated light beam resulting in the presence of a dark spot (zero fluorescent intensity). Immediately after the bleaching process, the bleached molecules are finally substituted by the unbleached molecule outside the bleached area due to the Brownian motion (Meyvis et al. 1999). As a result, the recovery rate of the fluorescent intensity measuring in the bleached area can be observed from which the diffusion coefficient of the fluorescent molecules can be extrapolated. Here the FRAP technique was used for measuring the diffusion of intrinsically fluorescent poly-lysine dendrimers.

3.2 Materials and methods

3.2.1 Materials

Amino latex nanospheres (ALN) (108±7 (±SD) and 495±23 nm) and carboxyl latex nanospheres (CLN) (48±7, 91±9.8 and 483±10 nm) were purchased from Polysciences Inc., USA. Actin from rabbit muscle, sodium azide, Trisma® hydrochloride solution, pH 7.4, Adenosine 5’-triphosphate (ATP) disodium salt, Magnesium chloride, imidazole, D-(+)-Glucose, Hydroxypropyl methyl cellulose (HPMC), MW 22,000 Da, ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA) and potassium chloride were purchased from Sigma, USA. 1,4-Dithio-DL-threitol (DTT) was purchased from Fluka, Switzerland. FluoSpheres® carboxylate-modified microspheres, 100nm, yellow-green fluorescent were purchased from Molecular Probes, Netherlands.

3.2.2 Methods

3.2.2.1 Effect of concentration on diffusion coefficient

Aqueous suspensions contained various concentrations of 50 nm Fluoresbrite® carboxyl NYO microspheres, 0.02-0.1% (w/v), in 25%, 12.5%, 6.25%, 3.125% and 1.5625% (v/v) glycerol solutions were prepared using double distilled deionised water as solvent. The viscosity of the glycerol solutions was determined using an Anton Paar microviscometer (AMVn, No. 670098). Refractive indices were measured (Bellingham & Stanley Ltd, refractometer, London) at room temperature.
The same series of concentrations of microsphere suspensions was prepared in 12.5% (v/v) glycerol solution. Both series were filtered through a 0.22 micron membrane filter before measuring the diffusion coefficient by PCS, at a wavelength of 633 nm at 25°C. All the experiments were carried out in triplicate.

### 3.2.2.2 Effect of viscosity of media on diffusion coefficient

Amino latex nanospheres (ALN) (108±7 (±SD) and 495±23 nm) and carboxyl latex nanospheres (CLN) (48±7, 91±9.8 and 483±10 nm) (Polysciences) were used. AR glycerol obtained from BDH was used after centrifugation to remove any dust particles. The aqueous suspensions contained 0.2% (w/v) of either 50 nm positively or negatively charged latex nanospheres and 0.02% of the 108 and 495 nm positively and negatively charged latex nanospheres were prepared in glycerol aqueous solutions, 0-25% (v/v), using double distilled deionised water. All of these samples were filtered through 0.22 micron filters except the sample containing 495 and 483 nm nanospheres which were filtered through a 0.9 micron membrane filter prior to measuring the diffusion coefficient by PCS, at a wavelength of 633 nm at 25°C. The viscosities and the refractive indices of glycerol aqueous solutions were determined as mentioned in 3.2.2.1.

### 3.2.2.3 Diffusion of nanospheres in HPMC gels

For studies with HPMC gels, a 2.0% (w/v) gel stock was prepared in cold double distilled deionised water and the polymer was allowed to swell in water for 24 h until a clear state was reached. Various gels containing 108 nm positively and 91 nm negatively charged nanospheres, 0.02% (w/v), were prepared. The bulk viscosities of the gels were measured using a CarriMed CSL 500 Rheometer with 4 cm, 2 degree cone at 25°C. The samples were filtered and the diffusion coefficients were measured under the conditions mentioned in 3.2.2.1.

### 3.2.2.4 Diffusion study of dendrimers using FRAP method

#### 3.2.2.4.1 Dendrimer synthesis

The details of the synthetic procedure and purification of the 6<sup>th</sup> generation polyllysine dendrimer, (Gly)(Lys)<sub>63</sub>(NH<sub>2</sub>)<sub>64</sub>, are described in Chapter II. When the last
coupling was completed, the compound was cleaved from the resin by HF and precipitated with diethyl ether. The precipitated dendrimer was separated from the resin by filtering. The compound was washed with 20% glacial acetic acid or water and freeze-dried, dried over P₂O₅ for three days and stored over silica gel. The compound was purified by exhaustive dialysis against deionised water using pleated dialysis tubing (Snakeskin™, 3500 Da MWCO, 22 mm dry diameter (ID)).

3.2.2.4.2 System validation

The standard curve of the fluorescent nanospheres, 100 nm, with maxima excitation and emission of 505 and 515 nm, and the 6th generation fluorescent polylysine dendrimers were made. Various concentrations ranging from 0.0625-2.0 % (w/v) of the fluorescent nanospheres and 0.225-3.6 % (w/v) of the dendrimers were prepared in PBS. The sample of each concentration, 25 µl, was placed into a cover well image chamber with a diameter of 13 mm and 0.2 mm depth (Figure 3.1). The samples were then viewed under the confocal microscope and the fluorescent intensity of all the samples at the same depth in the same defined geometry were measured and recorded with fixed detector gain, amplifier offset, amplifier gain and laser power. The excitation and emission wavelength of the dendrimers are 488 and 515 nm respectively. The relationship between the fluorescent intensity of the sample and the concentration of sample solutions were plotted. The linear relationship between those two parameters was identified and the working concentration of the dendrimers and the fluorescent nanospheres were selected.

![Figure 3.1](image)

**Figure 3.1** The sample solution of the fluorescent nanospheres or the dendrimer was placed into the cover well image chamber. Firstly, the protective liner from the cover well gasket was peeled off (A). The gasket was placed on coverslip with the open-chamber side up (B). The sample solution, 25 µl, was filled into the well (C). Finally the microscope slide was placed over the adhesive surface of the gasket and pressed gently but firmly on microscope slide to seal the chamber (D).
3.2.2.4.3 Diffusion of nanoparticles and dendrimer in PBS and glycerol solution

The selected concentration (from 3.2.2.4.2) of the fluorescent nanospheres and the dendrimers were prepared in PBS and in various concentration of glycerol solution, 20-60% (v/v) using PBS as the solvent. The samples were placed into the cover well image chamber as described in 3.2.2.4.2. The diffusion of the nanospheres and the dendrimers were evaluated using FRAP (Braeckmans et al. 2003). A number of assumptions are required for this FRAP model. First, the sample should have a uniform distribution of the fluorescent molecules in the samples and thus the diffusion process is taking place in an isotropic and infinite medium. Next, the lens with low number of aperture (NA) is recommended for observation in 3-D. The bleaching duration should be at least 15 times shorter than the duration of the characteristic recovery process. Lastly, there should be no flow in the medium.

Briefly, in the confocal microscope software (Zeiss LSM 510) a single track (mode of operation) was used with optical lens of 10x (NA = 3.0). The region of interest in the sample was brought into focus with an entirely open diaphragm to perceive the highest fluorescent light and minimize bleaching during the fluorescent recovery process. Inside the bleaching control window, the bleach geometry and control geometry in the same size circular disc with a particular diameter was drawn; the time intervals and the number of the intervals were specified to get an image series representing the disc before bleaching (in the first image), at the bleaching period (in the second image) and the fluorescent recovery process (the following images) in order. Time intervals and the number of the intervals are assigned according to a minimum time interval (100 ms) and the recovery speed. Practically, there are 30-40 images in each image series.

The bleaching experiment was performed and the data were recorded. The images were saved as raw data series and were converted into the multi-page tif file type using IrfanView software (version 3.92). The data were then analysed by the Image processing software (version 2.3) written in Matlab (version 6.5) by Dr Kevin Braeckmans (Ghent university, Belgium). The data were extracted as the following. The bleached area was identified by a center-of-mass algorithm. The recovery images
were normalized to the prebleach image. The noise occurring during analysis process was excluded using a 3x3 convolution mask. Then the mean fluorescent intensity within the bleached area was appraised and was normalized to that value obtained from the background in control disc. The experimental parameters were then extracted using least-squares fit (Braeckmans et al. 2003).

In order to achieve the diffusion coefficient with the highest possible accuracy some assumptions that are to be met by the experimental conditions were assigned as the follows (Braeckmans et al. 2003).

a) In the specimen the fluorescent molecules are expected to be initially uniform distribution. Meaning that the concentration gradient is not allowed to present in the system before bleaching.

b) The diffusion process is presumed to be isotropic system which takes place in an infinite medium. This means that during the recovery period the diffusion front should not reach the boundaries where it will be reflected and influence the random diffusion process. Practically, the area that is adjacent to the edge of the sample chamber should not be selected to perform the experiment.

c) To create the 3-D diffusion system an objective lens with low numerical aperture of the objective (NA) should be selected for bleaching and observation the fluorescent recovery because it possesses a cylindrical illumination profile. Lenses with the higher NA have a conical profile but it can be used if the thickness of the sample is minute compared to the axial resolution of the lens. However the diffusion in this case (high NA) is limited to the 2-D diffusion.

d) Bleaching period must be sufficiently short to circumvent fluorescent recovery during bleaching. This signifies that the total bleaching period should be at least 15 times smaller than the recovery period.

e) Flow of the fluorescent molecules within the specimen is prohibited as it can contribute to the fluorescent recovery, and this can be checked physically by examining the position of the bleached disk in recovery image series. The checking can be confirmed by the value of two parameters: the mobile fraction.
(k_{fit}) and the bleaching parameter (K_{fit}) which can be obtained from the least squares curve fitting. To attain the diffusion coefficient (D_{fit}) with the highest possible accuracy k_{fit} and K_{fit} must be 0.97 \leq k_{fit} \leq 1.03 and 0 \leq K_{fit} \leq 6, respectively.

3.2.2.4.4 Diffusion of dendrimer in actin gel

Actin stock solution, 2.5 mg/ml (20\mu l) and Alfa actinin solution, 2.5 mg/ml (5\mu l) were added into buffer G which contained 0.2 mM adenosine 5'-triphosphate (ATP), 0.5 mM dithiothreitol (DTT), 0.2 mM calcium chloride, 1 mM sodium azide and 2 mM Tris-HCl, pH 8.0. The dendrimer at a concentration of 3.6% (w/v), 14 \mu l, was added and the solution was mixed well. Finally, actin was polymerized by mixing 9 volumes of actin stock solution and 1 volume of polymerization buffer (buffer P) which contained 500 mM potassium chloride, 10 mM magnesium chloride, 10 mM EGTA and 100 mM of imidazole, pH 7.0. The gel was allowed to polymerize overnight at room temperature in a cover well image chamber gasket (Figure 3.1). The sample was placed in the confocal microscope stage and the diffusion of the dendrimers was determined using the FRAP method as described in 3.2.2.4.3.

3.3 Results and discussion

3.3.1 Effect of concentrations on diffusion coefficient

Figure 3.2 demonstrates the diffusion coefficient (D) of nanospheres both in water and in 12.5% (v/v) glycerol. It is seen that the diffusion coefficient of nanospheres in water was, as expected, higher than that in 12.5% glycerol (Stokes-Einstein equation). The increase in concentration of nanospheres only resulted in a slightly changed value of diffusion coefficient in both water and glycerol. In water, at a concentration of 0.02% (w/v) nanospheres, the diffusion coefficient of nanospheres was 6.93 \mu m^2/s (or 6.93 \times 10^{-8} \text{ cm}^2/\text{s}). Then the diffusion coefficient increased to 7.12, 7.18 and 7.26 \mu m^2/s when the concentration of nanospheres was increased to 0.04, 0.06 and 0.08% (w/v) before declining to 7.06 \mu m^2/s at a concentration of 0.1% (w/v). A similar pattern was found in 12.5 % glycerol as can be seen in Figure 3.2. The diffusion
coefficients were found to be between 5.00-5.05 \( \mu m^2/s \) when the concentrations of nanospheres were in the range from 0.02-0.1\% (w/v).

![Graph showing diffusion coefficients in water and 12.5\% (w/v) glycerol](image)

**Figure 3.2** Diffusion coefficients of 50 nm nanospheres at various concentrations in water and in 12.5\% (v/v) glycerol showing the lower diffusion coefficient in glycerol solutions.

### 3.3.2 Effect of viscosities of media on diffusion coefficient

The diffusion coefficients of latex nanospheres with diameter from 48-500 nm are illustrated in **Figure 3.3**. It was not surprising that in water and Newtonian glycerol aqueous solution, \( D \) values obey Stokes-Einstein behaviour over a range of system viscosities. This was also confirmed by the linear relationship between the measured diffusion coefficient \( D \) and the reciprocal of viscosity found in **Figure 3.4**, as predicted by the Stokes-Einstein equation. **Figure 3.5** shows the theoretically estimated values \( (D_{theo}) \) of the diffusion coefficients versus those actually measured \( (D_{exp}) \). The particle sizes of nanospheres used in this experiment have a small size distribution. From the standard deviation values of nanosphere diameter, the percentage of the maximum error limit for \( D_{theo} \) was calculated and used to evaluate the \( D_{exp} \) values. The solid line corresponds to equation 3.9 and the dashed line shows the acceptable deviation limit (10\%) from the solid line. There was a good correspondence between the measured values and their theoretical estimates for 91 and 483 nm CLN and 495 nm ALN. As can be seen, all measured diffusion
coefficients ($D_{\text{exp}}$) of 91 nm CLN fell in the 5% error limit while that of 495 nm ALN and 483 nm CLN fell in the 10% error limit. However, most of the $D_{\text{exp}}$ values of 48 nm CLN and 108 nm ALN were found in between the 10% and 30% error limit which implied an inaccuracy and constraint of the PCS for this application. No effect of particle charge on the diffusion coefficient was found in this study as can be seen from Figure 3.6.

![Figure 3.3](image.png)

**Figure 3.3** Diffusion coefficients of positively (ALN) and negatively charged nanospheres (CLN) (0.2% w/v of 48 nm CMS and 0.02 % w/v of 108 and 495 nm ALN and 91 and 483 nm CLN), in various concentrations of aqueous glycerol solutions.

![Figure 3.4](image.png)

**Figure 3.4** This graph illustrates the linear relationship between the diffusion coefficient ($D$) and the reciprocal viscosity as predicted by the Stokes-Einstein equation.
Figure 3.5 The parity plot shows the measured diffusion coefficients ($D_{\text{exp}}$) versus the estimated values ($D_{\text{theo}}$) of positively (ALN) and negatively charged nanospheres (CLN) (0.2% w/v of 48 nm CMS and 0.02% w/v of 108 and 495 nm ALN and 91 and 483 nm CLN) in various concentration of aqueous glycerol solutions. The solid line has been calculated from equation 3.9 ($D_{\text{theo}}$) and the dashed lines are $\pm 10\%$ of $D_{\text{theo}}$. 
Figure 3.6 The relative diffusion coefficients of positively (ALN) and negatively charged nanospheres (CLN) (0.2% w/v of 48 nm CMS and 0.02 % w/v of 108 and 495 nm ALN and 91 and 483 nm CLN) in various concentrations of glycerol solutions.

3.3.3 Diffusion of nanospheres in HPMC gels

An increase in the bulk viscosity of the HPMC gel resulted in the decline in diffusion coefficient and relative diffusion coefficient \( (D/D_0) \) of the nanospheres (Figure 3.7-3.9). It was found as expected that the smaller the particles the faster they diffuse in the gels (Figure 3.7 and 3.8). Figure 3.8 illustrates the deviation of \( D \) values from the Stokes-Einstein relationship; a linear relationship was not found between the diffusion coefficients and the reciprocal viscosity of HPMC gels. There was a slight effect of particle charge on the diffusion coefficient as shown by the lower relative diffusion coefficients of the negatively charged nanospheres (Figure 3.9). Nonetheless, it was seen from Figure 3.7 and 3.9 that the effect of particle size is far more prominent than the effect of charge of the particles. Furthermore, it was observed that the polydispersity index (PI) of systems containing the positively charged nanospheres increased dramatically from 0.217 at HPMC concentrations of 0.2 % (w/v) to 0.495, 0.768 and 0.928 when the concentration of HPMC was 0.4%, 0.6% and 0.8 % (w/v), respectively. A similar trend was not observed in the case of negatively charged nanospheres where the PI was found to be in the range of 0.035-0.470 (HPMC
concentration range 0.2% to 0.8%, w/v). These results might suggest an interaction between the nanospheres and the polymer, although, no evidence of the interaction has been found from Figure 3.9. In addition, these results also indicated that there is a certain limit on the condition (viscosity and types of medium) of the samples which their diffusion coefficients would be accurately determined by PCS. Further work is required on this.

In gels as discussed earlier because of the exclusion effect and obstruction effect, the bulk viscosity does not determine the diffusion of solutes in gels but rather it is the "microscopic" or "effective" viscosity which does. The effective viscosities of the HPMC gel at various concentrations were calculated. The effective viscosities ($\eta_e$), calculated using equation 3.10 were found to be 0.899, 0.908, 0.917 and 0.926 mPa.s at HPMC concentration of 0.2%, 0.4%, 0.6% and 0.8 % (w/v) respectively (Table 3.1). Interestingly, at low concentrations of HPMC gel, the effective viscosity ($\eta_e$) was close to the viscosity of water (0.890mPa.s). The value of $\eta_e$ increased with the increase in the concentration of HPMC as might be anticipated with increased water of hydration or the protrusion of portions of the macromolecular chains increasing the effective viscosity of the aqueous channels. From the effective diffusion ($D_e$) of the nanospheres calculated from equation 1.10, the results implied that the diffusion coefficient of the particles in HPMC gel is dependent on the volume fraction of the HPMC in the gel. The diffusion rate of the nanospheres in 0.2, 0.4, 0.6 and 0.8 % w/v HPMC gels were found to be 1%, 2%, 3% and 4% slower than that in water, respectively. From Table 1 the experimental viscosity of pure solvent (water) was extrapolated from the interception of the plot between the $\eta_e$ values and HPMC concentration. The values of microscopic viscosity determined by extrapolation to zero percentage HPMC is 0.891mPa.s which is the viscosity of water at 25 °C.
Figure 3.7 Diffusion coefficients of 108 nm positively (ALN) and 91 nm negatively charged nanospheres (CLN) (0.02 % w/v of ALN and ALN), in various concentrations of HPMC gels.

Figure 3.8 This figure shows the correlation between the diffusion coefficient of 0.02% (w/v) 108 nm positively and 91 nm negatively nanospheres and the reciprocal viscosity of HPMC gels.
**Figure 3.9** Graph demonstrates the relative diffusion coefficients of 0.02% (w/v) dispersion of 108 nm positively and 91 nm negatively nanospheres in various concentrations of HPMC gels as a function of viscosity (mPa.s).

**Table 3.1** The calculated effective viscosities of HPMC gels

<table>
<thead>
<tr>
<th>Concentration of HPMC % (w/v)</th>
<th>Density (g/ml)</th>
<th>Volume fraction (φ)</th>
<th>$\frac{1-\phi}{1+\phi}$</th>
<th>$(1-\phi)^2$</th>
<th>$\eta_e$ (mPa.s)</th>
</tr>
</thead>
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<tr>
<td>0.2</td>
<td>0.9945</td>
<td>0.0020</td>
<td>0.9960</td>
<td>0.9900</td>
<td>0.899</td>
</tr>
<tr>
<td>0.4</td>
<td>1.0031</td>
<td>0.0040</td>
<td>0.9921</td>
<td>0.9803</td>
<td>0.908</td>
</tr>
<tr>
<td>0.6</td>
<td>1.0242</td>
<td>0.0058</td>
<td>0.9884</td>
<td>0.9713</td>
<td>0.917</td>
</tr>
<tr>
<td>0.8</td>
<td>1.0276</td>
<td>0.0077</td>
<td>0.9847</td>
<td>0.9621</td>
<td>0.925</td>
</tr>
</tbody>
</table>
3.3.4 Diffusion study of dendrimer using the FRAP method

3.3.4.1 System validation

The calibration curves exhibit the correlation between the fluorescent intensity (F) of the 100 nm fluorescent nanospheres and the 6.5 nm intrinsic fluorescent dendrimers and their concentrations are, respectively, illustrated in Figure 3.10 and 3.11. The correlation coefficients (r) are 0.9998 and 0.9997, respectively. A similar trend was observed when the concentrations of the nanospheres and the dendrimers were increased. For the fluorescent nanospheres (Figure 3.10), the linear relationship between the F value and the concentrations was found at nanosphere concentration between 0.0625 and 0.75% (w/v), yet the deviation appeared after the concentration of the nanospheres was increased to 1 and 2% (w/v). This result reflected the general property of a fluorescent molecule and the limitation of the confocal microscope that the fluorescent intensity can be found linearly increasing with fluorophore concentration until reaching saturation range. To quantify the fluorescence, the measurement should be performed within this linear region. The concentration of the fluorescent nanospheres at 0.25% was, therefore, selected as working concentration in the next experiment to exclude the experimental error.

For the dendrimers, a linear relationship between the F value and the dendrimer concentration was found at all concentrations (Figure 3.11). The fluorescent intensity range detected from the dendrimer is lower than that of the nanospheres. An increase in concentration of the dendrimers resulted in an increase in fluorescent intensity. In other words, the fluorescence of the dendrimer is concentration dependent, albeit the fluorescence of the dendrimer is intrinsic even though lacking fluorophores in its molecular structure (Al-Jamal et al 2006). However, the saturation of the fluorescent intensity was also found at a higher concentration of the dendrimer (data not show here). The difference may be due to the dissimilarity in molecular structure between the dendrimer and the nanospheres. The dendrimer concentration of 1.8% (W/V) was selected as a working concentration for the diffusion study.
Figure 3.10  This calibration curve represents the relationship between the fluorescent intensity (F) and the concentration of the fluorescent nanospheres (%, w/v) in PBS. The correlation coefficient (r) is 0.9998.

Figure 3.11  The calibration curve exhibits the relationship between the fluorescent intensity (F) and the concentration of the dendrimers (%, w/v) in PBS. The correlation coefficient (r) is 0.9997.
3.3.4.2 Diffusion of nanospheres and dendrimers in PBS, glycerol solution and in actin gel

The results of a FRAP experiment to determine the diffusion of the fluorescent nanospheres, 100 nm in diameter, in PBS is presented in Figure 3.12. The stacked images in a grey scale demonstrate the specimen during the fluorescent recovering process (A). The images were scanned with a time interval of 0.801s over 30 intervals. Two circular discs are assigned as the bleaching geometry (red arrow) and the control geometry (blue arrow). The first image is the prebleached image at time t=0 s. This can be explained using a fundamental aspect of the FRAP method that the fluorescent nanospheres inside the bleaching geometry were, first, bleached with a highly attenuated laser beam. The high energy initiated bleaching reaction of fluorescent nanospheres inside the fluorescent nanospheres resulting in a vanishing fluorescent signal of the fluorescent molecules. The next image shows the specimen when the nanospheres were bleached and the following images show the recovery process of the specimen due to the Brownian motion of the fluorescent particles. Bleached particles were replaced by unbleached particles from out side the bleached area. This can be seen from the ascending fluorescence in the bleaching geometry.

The repopulation rate of fluorescent nanospheres was measured and processed using an image processing program written in Matlab (as mention in method section 3.2.2.4.3) as show in Figure 3.12B. The diffusion coefficient (D) and other experimental parameters were extracted from the experimental data (blue dot) by least squares fitting (in red line) as can be seen in Figure 3.12B. The \( k_{\text{fit}} \) and \( K_{\text{fit}} \) are mobile fraction and the bleaching parameter as defined under 3.2.2.4.3 in page 76-77.

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**Figure 3.12 (over page, p87)** The diffusion coefficient (D) of 100 nm fluorescent nanospheres in PBS was measured using the FRAP technique. The serial images in grey scale demonstrate the specimen during the experiment was performed (A). There are two circular discs representing the bleaching geometry (red arrow) and the control geometry (blue arrow); the control region is not bleached. The first image was taken before bleaching at time t=0s (Prebleach image). The second image shows the specimen when it was bleached and the following images show the recovery process of the specimen due to the Brownian motion of the fluorescent particles. The images were scanned with time intervals of 0.801s for 30 intervals. In B, the data have been processed using an image processing program written in Matlab (as mention in method section 3.2.2.4.3). The diffusion (\( D_{\text{fit}} \)) and other experimental parameters (\( k_{\text{fit}} \) and \( K_{\text{fit}} \) were defined under 3.2.2.4.3) were extracted from the experimental data (blue dot) by least squares fitting (in red line) (B).
Note: see caption in the previous page.
The diffusion of the 100 nm nanosphere and the 6.5 nm dendrimer are illustrated in Figure 3.13-3.14. The FRAP technique can measure the effect of viscosity of media on the diffusion coefficient at higher concentrations of glycerol, 0-60% (v/v), than that can be measured by the PCS technique, 0-25% (v/v) glycerol. This may be due to the limitation of the dynamic light scattering system. Although the concentration range of the glycerol was extended up to 60% (v/v), the diffusion coefficients ($D$) of both the nanospheres and the dendrimers comply with the Stokes-Einstein relation. This was reinforced by the linear relationship between the measured diffusion coefficients and the reciprocal viscosity found in Figure 3.13 and 3.14 as predicted by the Stokes-Einstein equation. The $k$ value indicated in Figure 3.13 implied the fitting quality of the experimental data. All the accepted data require that this $k$ value be within 3% error range (0.97-1.03) from the ideal value that equal to 1. The $k$ value of the nanospheres and the dendrimers were found to be 0.99-1.01 and 1.01-1.02, respectively. The $D$ values of the 100 nm nanospheres and the dendrimers in PBS were 3.38±0.20 (SD) and 79.35±1.22 (SD) μm$^2$/s, respectively.

An increase in the viscosity of the glycerol resulted in the decline in the relative diffusion coefficient ($D/D_{PBS}$) of the nanospheres and the dendrimers (Figure 3.15). The relative diffusion coefficients decreased with an increasing glycerol concentration and at the same concentration of glycerol the relative diffusion coefficient of the nanospheres is higher than that of the dendrimers. The theoretically estimated value ($D_{theo}$) of the diffusion coefficients versus those experimental values measured by FRAP method ($D_{exp}$) of both the nanospheres and the dendrimers are illustrated in Figure 3.16. Since the particle size of the nanosphere used in this experiment possesses a small size distribution, the standard deviation of the nanosphere diameter can be used to calculate the maximum error limit of the $D_{theo}$, a value subsequently used to evaluate the $D_{exp}$ values. The solid line represents the $D_{theo}$ values calculated from equation 3.9 and the dashed line represents the acceptable deviation limit (3%) from the solid line. There was a good agreement between the measured values and their theoretical estimates for both the 100 nm fluorescent nanospheres and the 6.5 nm dendrimers. This was confirmed by all measured diffusion coefficients ($D_{Exp}$) of the
nanospheres and the dendrimers falling within the 3% error limit which implied an accuracy and precision of FRAP method for this application.

Figure 3.13 The diffusion coefficients of the nanospheres in various concentrations of glycerol solutions and the k values are used to indicate the fitting quality of the experimental data and system validation. The k value is a mobile fraction and it is valid if all molecules in the system are random mobiles and thus there is no flow occurring in the system. In this case, from the least squares fitting the k values lie within an acceptable limit range (0.97-1.03) demonstrating the system is validated.
Figure 3.14  The diffusion coefficients of a dendrimer with 6.5 nm diameter in various concentrations of glycerol solutions, plotted here as a functional of reciprocal viscosity.

Figure 3.15  The relative diffusion coefficient of a 6.5 nm dendrimer and a 100 nm nanosphere in various concentrations of glycerol solutions
The diffusion coefficient of the 6.5 nm dendrimer in actin gels measured by the FRAP method was found to be $23.75 \pm 3.22$ (SD) $\mu$m$^2$/s. The relative diffusion coefficient of the dendrimers ($D_{\text{act}}/D_{\text{PBS}}$) was found to be 0.30. This suggested that the construction of actin network inside the actin gel could hinder the mobility of the dendrimers up to 75% compared to PBS. Actin bundles are dominantly found inside cell cytoplasm and may play an important role on the mobility of the nanocarrier systems. The viscosity of the cytosol is said to be 2.6 to 10 fold higher than water; the variation perhaps due to the differences in cell types and different techniques (Luby-Phelps et
al. 1986; Luby-Phelps et al. 1987; Kao et al. 1993; Swaminathan et al. 1996; Swaminathan et al. 1997; Seksek et al. 1997; Partikian et al. 1998). These viscosity values have contributed to an understanding of the retarded diffusion, although the medium in which particles diffuse in approximates pure water (Fushimi & Verkman 1991; Kao et al. 1993). The dissimilarity between the relative diffusion coefficients of the dendrimers found in actin gel and in viscosity of the cytosol (see Chapter IV) might be due to the lower actin concentration used in an in vitro actin gel preparation. In fact the concentration of the actin is evidently found up to 10 mg/ml (dos Remedios & Thomas 2001). In the case of the dendrimers, there is no significant difference between the diffusion values of the dendrimers in water and in PBS. To be able to compare this system to the cell cytoplasm is a prelude to the development of effective carrier systems.

3.4 Conclusion

The diffusion coefficients of 48-500 nm positively and negatively charged latex nanospheres in water (Newtonian and isotropic systems) could be predicted as anticipated by the Stokes-Einstein relationship. By using photon correlation spectroscopy (PCS), the diffusion coefficient of these latex nanospheres could be rapidly measured. However when the viscosity of this system was increased by adding various concentration of glycerol, PCS as a method might become inaccurate. The reason for this is still not clear but we have assumed that it might be due to the higher viscosity of the media limiting the capability of PCS system.

In HPMC gels which are pseudoplastic and non-isotropic systems, the diffusion coefficients of the nanospheres were found to deviate from the Stokes-Einstein relationship. Owing to the apparent viscosity or bulk viscosity of the gels could not be used to determine the diffusion coefficient; we therefore calculated the micro-viscosity of the gels. Assuming the hindrance of the mobility of the particles inside the gels could be quantitatively attributed to the void fraction that is accessible to the diffusing solute effective ($\varepsilon$) and a tortuosity factor ($\tau$) - well known as the exclusion effect and obstruction effect. Unfortunately these values could not be measured directly from the experiments. Therefore using the polymer volume fraction, $\varphi_p$, to
predict the effective diffusion coefficient is preferable and following the equation of Mackie and Meares (1955) the diffusion of nanospheres in various concentration of HPMC gel could be calculated. The results implied that the diffusion coefficient of the particles in HPMC gels is dependent on the volume fraction of the HPMC in the gel, for example, in HPMC gels (0.2, 0.4, 0.6 and 0.8 % w/v) the diffusion coefficient of the latex nanospheres was found to be only 1%, 2%, 3% and 4% slower than that in water, respectively.

In contrast to PCS, under conditions of high salt and polymer concentrations, fluorescence recovery after photobleaching can be used as a powerful tool for studying the dynamic mobility of the nanoparticles. The diffusion of nanoparticles and dendrimer can be accessed efficiently, albeit the glycerol concentration in the media was increased up to 60% (v/v). This FRAP model can also be used to access the diffusion coefficient of the dendrimers in heterogeneous media such as an actin gel and biological fluids while preserving their original properties. The diffusion coefficient of the dendrimer in actin gel (23.75 μm²/s) was found reducing by approximately 75% to that in PBS (79.35 μm²/s). The concentration of actin found in cells (10 mg/ml) is higher than what we used here; the diffusion of the dendrimer in cells is, therefore, expected to be lower than our values. The competent comparison between this in vitro actin gel and the cytosol is a prologue to the development of effective carrier systems. Good models of the physical structure of cell interiors are required, along with probes such as the one described here to advance the design of new delivery vectors. This will be discussed in the next Chapter V and VI.
Chapter IV

FORMULATION AND TRANSFECTION STUDIES OF DENDRIMER-DNA COMPLEXES (DENDRIPLEXES)

Summary

The cationic symmetric poly-lysine dendrimers (DM1), (Gly)(Lys)$_{63}$(NH$_2$)$_{64}$, and amphipathic poly-lysine dendrons (DM2), (C$_{18}$)$_3$(Lys)$_7$(NH$_2$)$_8$, are treated here as gene delivery vehicles. The complexes between dendrimers/dendrons and DNA, so-called “dendriplexes”, were physicochemically characterised by determination of particle size, shape and zeta potential. Dendriplexes appear in spherical, toroidal, oval and rod-like structures in TEM. The plasmid DNA (pDNA) was completely condensed at molar charge ratios of 5:1 (+/-) for both DM1 and DM2. This was confirmed by the results from agarose gel retardation assays. The diameter of the dendriplexes ranged between 73-519 and 61-107 nm for the DM1- and DM2-DNA complexes, respectively. The surface charge of the dendriplexes became positive when the molar charge ratios of the dendrimers and dendrons exceeded or equaled to 4:1 and 2:1. Both types of the dendrimers exhibited relatively high cytotoxicity in all the four observed cell lines: Caco-2, HEK-293, SKMES-1 and REFS-2 cells, so studies were conducted at concentrations where toxicity was minimised. Fifty percent inhibitory concentrations (IC$_{50}$) of the dendrimers ranged from 3.7 to 7.5 mg/ml. Both the DM1 and DM2 dendrimers could enhance transgene expression over naked DNA in HEK-293 and Caco-2 cells.
4.1 Introduction

Deoxyribonucleic acid (DNA) has become a vital therapeutic material for incurable diseases including cancer and hereditary infirmity. This polyanionic molecule is too large to penetrate cell membranes and unstable in the extracellular milieu (Ledley & Ledley 1994). Proficient DNA delivery is, therefore, problematic. Ideally, the DNA has to be compacted into a particular size range which permits it to be selectively taken up by target cells. The condensed DNA should be stable and be able to withstand any harsh environment, and also be able to overcome different cellular barriers. In addition, the negative charge of the DNA has to be overcome to circumvent its irrelevant interaction with other proteins during transportation (Zauner et al. 1998). Importantly, the DNA must be released inside the target cells and be allowed to voyage to the cell nucleus so providing high transgene expression.

Watson and Crick (1953) proposed that DNA is composed of two helical strands of polynucleotides which are held together by hydrogen bonds between the nucleotide subunits of each strand. Each strand consists of nucleotide subunits and sugar-phosphate backbones. In nature, the DNA is packed inside chromosomes which locate inside the nucleus in order to preserve biological activity. The major proteins necessitated for this packing process for DNA are histones. However the nature of the interaction between DNA and histones remained unclear until the high-resolution structure of chromatin was envisaged in 1997 (Luger et al. 1997). A high proportion of positively charged amino acids (lysine and arginine) allow the histones to bind tightly to the negatively charged sugar-phosphate backbone of DNA. These copious interactions explain, in part, why practically any sequence of DNA can bind to a histone core. There is also a long N-terminal amino acid “tail” in each histone core which extends out from the DNA histone complex. These tails are responsible for several types of covalent modification that control an appearance of chromatin structure.

Since DNA is a macromolecule that, per se, is not internalized by cells and thus vectors are required to improve its delivery. There are, hitherto, three regular approaches for DNA delivery: viral vectors, non-viral vectors and physical
transfection (e.g. electroporation, DNA injection, microinjection and particle bombardment). Although the use of viral vectors exhibits high gene expression, it is difficult to be reproducibly conducted in a large scale and may cause deleterious effects such as inflammatory, immunogenicity and secondary malignant induction (Lattime 2002). For physical transfection, an interesting result obtained by Page et al. (1995) demonstrated that there was no transgenesis when naked DNA was injected into the cell cytoplasm. In fact, there was also evidence of the sequestration of the naked DNA and cytoplasmic elements which precluded transgene expression. Furthermore, the percentage of transgenesis by physical transfection was also dependent on temperature, cell type and distance from the nucleus to injection site. However, an injection of naked DNA to exhibit local transient transgene expression may be achievable (Wolff et al. 1990). To create a systemic effect by this technique is rather difficult because intravenous injection of naked DNA provides low levels of gene expression in all major organs (Liu et al. 1995). These findings resulted in an attempt to develop non-viral vector gene carrier systems.

Ideal materials which can condense and protect DNA have been searched for. Histones and poly-lysine were the very first materials to be tested as non-viral vectors, because histones are necessary for the packing process of the DNA inside chromosomes, as discussed. Lysine amino acid is abundantly found in histone molecules. However, its cytotoxicity has been found. Both compounds have the ability to condense DNA and enhance transgene expression (Fritz et al. 1996; Zauner et al. 1998). There was 12.5% transgenesis when the complex of DNA and poly-lysine was injected into the cell cytoplasm in comparison to naked DNA (Page et al. 1995). This result was reinforced by the result obtained by Dowty et al (1995). These implied that poly-lysine could facilitate the migration of the DNA toward cell nuclei and eventually could accomplish transgene expression (Dowty et al. 1995). An intensive study of poly-lysine based gene carrier systems has been carried out and a review on this can be seen elsewhere (Männistö et al. 2002). To date, other competitive non-viral gene carrier systems have been invented such as cationic lipid-based carriers (Sakurai et al. 2000; Woodle & Scaria 2001; Sugiyama et al. 2004), cationic polymer-based
carriers (Gebhart & Kabanov 2001), dendrimers (Brown et al. 2001; Tomalia 2005) and nanotubes (Kateb et al. 2007).

Dendrimers are the topic of interest in this chapter in relation to their potential as a gene carrier system (Dufes et al. 2005; Tack et al. 2006), although dendrimers in general have been exploited as image contrast agents, nuclear imaging and in cancer radiotherapy (Mitra et al. 2006). In our research group, various types of lysine-based dendrimers have been synthesised and their transfection efficiencies have been evaluated (Sakthivel et al. 1998; Shah et al. 2000). The physicochemical properties of dendriplexes of poly-lysine based dendrimers have been characterised (Ramaswamy et al. 2003a; Ribeiro et al. 2005). In this present study, two types of dendrimers (symmetric and partial polylysine dendrimers) were used to formulate dendriplexes. Their ability to condense DNA, their physicochemical properties, toxicities and transfection abilities have been measured. The results are presented in this chapter.

4.2 Materials and methods

4.2.1 Materials

Poly-L-lysine hydrobromide (Mw 9,600, 23,800 and 121,000 Da), agarose (molecular biology grade), Trisma® base, boric acid, ethylenediamine tetraacetate (EDTA), 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), boric acid and ethidium bromide were purchased from Sigma, UK. Endofree Plasmid Giga kit was purchased from Qiagen Ltd, UK. Molecular weight marker, 1Kb DNA Ladder, sample loading buffer, Lipopectamine™ and Plus™ reagents were purchased from Invitrogen, USA. Plasmid DNA (pDNA) encoding gene expressing red fluorescent protein, pDsRed2-N1 (4.689 Kb) was purchased from Clontech, USA, respectively. A luciferase plasmid (7.2Kbp) and luciferase assay system (E4030) with reporter lysis buffer were purchased from Promega, USA. OptiMEM reduced serum medium, gentamicin solution (10 mg/ml), penicillin/streptomycin (100 unit), MEM non-essential amino-acids solution (MEM), Foetal bovine serum (FBS), phosphate buffer saline (PBS) (pH 7.4), Hank’s balanced salt solution (HBSS), Trypsin/EDTA were purchased from Gibco, UK. Mikrobiologie® and trypton soya broth were purchased
from Oxoid Ltd, UK and Merck, Germany, respectively. Citiflour® mounting medium were purchased from Molecular probes, Netherlands. Tissue culture flasks, 75 cm², with filtered screw caps were purchased from TPP, Switzerland. 96- and 12-well plates were purchased from Corning Costar Corporation, USA. Partial dendrimers (DM2), (C18)3(Lys)7(NH2)8, and its RGD conjugated form (DM3) were the gifts from Dr Chandra Ramaswamy, Centre for Drug Delivery (CDDR), The School of Pharmacy, University of London. Human colon adenocarcinoma cell line (Caco-2), Human embryonic kidney cell line (HEK-293), human Caucasian lung squamous carcinoma cell line (SKMES-1) and rat embryonic fibroblast cell line (REFS-2) were purchased from European Collection of Cell Cultures.

4.2.2 Methods

4.2.2.1 Extraction and purification of plasmid DNA (pDNA)

4.2.2.1.1 Plasmid amplification

An *E. coli* strain DH5α was used as a host for plasmid amplification. A single colony of *E. coli* containing the desired plasmid was picked from a freshly streaked selected plate and inoculated into 10 ml of starter culture medium, LB broth (Appendix II), containing 0.5 µg of kanamycin. The bacteria were grown for 8 h at 37 °C with vigorous shaking (approx. 300 rpm). Thereafter the starter culture was diluted 1:500 in 500 ml of the LB broth and the bacteria were allowed to grow for a further 12-14 h under the same conditions.

4.2.2.1.2 Plasmid purification

The plasmids were extracted and purified according to the manufacturer's instructions (Qiagen 2005) which are summarised in Figure 4.1. The method is based on an alkaline lysis technique and all details of related buffer can be found in Appendix II. Briefly, the bacteria were harvested (O) from the medium by centrifugation at 6000g for 15 min at 4°C. After vacuum filtration the bacterial pellets were resuspended in buffer (P1 buffer) containing 50 mM Tris hydrochloride buffer (pH 8.0), ribonuclease A (RNase A) and 10 mM of EDTA. Next the buffer containing 200 mM of sodium hydroxide (NaOH) and 1% sodium dodecylsulfate (SDS), P2 buffer, was added into
the resuspension. Appearance of RNase A in P1 buffer initiated cell digestion and cell
lysis to liberate DNA and RNA (2). Cell membranes composed of phospholipids and
proteins were then solubilised by SDS, thus promoting cell lysis. This allowed NaOH
(P2 buffer) to instigate the denaturisation of the chromosomal DNA and proteins
without penetration (3). The lysate was then counterbalanced (2) by adding an acidic
potassium acetate, 3.0 M, pH 5.5, (buffer P3) leading to SDS precipitation.
Simultaneously other cell debris (e.g. denatured proteins, cell membranes, organelles
and chromosomal DNA) except plasmid DNA were captured inside the precipitate.
The precipitate of cell debris was excluded by filtering through QIAfilter Mega-Giga
cartridge (4) and the pDNA was extracted using QIAgen-tip which exploited a
principal of ion-exchanged chromatography (5). The resin- a diethylaminoethanol
(DEAE) complex- is selectively bound to the pDNA, and thus all other impurities
were eradicated and eluted by QC buffer, containing 1.0M sodium chloride (NaCl), 50
mM 3-(N-morpholino) propanesulfonic acid (MOPS) pH 7.0, and 15% iso-propanol.
The pDNA was then eluted by QN buffer containing 1.25M NaCl, pH 8.5, and
precipitated by 0.7 volumes of isopropanol (6). The suspension was immediately
centrifuged at 15,000g, 4 °C for 30 min before the supernatant was carefully decanted.
The pDNA pellet was washed with endotoxin-free ethanol solution (70%, v/v) and
subsequently centrifuged at 15,000g, 4 °C for 30 min to collect the pDNA pellet (7).
Eventually, the pDNA pellet was air dried for 10-20 min and resuspended in a suitable
volume of endotoxin-free buffer TE (pH 8.0) or sterile water (8). The purified pDNA
must be kept at -80 °C until use.
Figure 4.1 DNA purification procedures in summary: *E. coli* are harvested by centrifugation (1), and their cell membranes and cell organelles are lysed to release DNA and RNA (2). Cell debris is precipitated (3) and removed by filtration (4) leaving pDNA inside the filtrate. The pDNA is extracted (5) and precipitated with isopropanol (6) before pelletisation (7) and resuspended in a suitable solvent (8). Details of all buffers used are listed in Appendix II.
4.2.2.1.3 Plasmid quantification

The pDNA was quantified using spectrophotometry (Beckmen 650 spectrophotometer). The fact that the nitrogenous base of the DNA exhibits a strong ultraviolet (UV) absorption at 260 nm and there is linear relationship between an absorbance of DNA solution at this wavelength and the DNA concentration are employed for measuring the concentration of pDNA. An extinction coefficient of DNA at 260 nm (E_{260}=20, in a 1 cm quartz cell) coincides with DNA solution at the concentration of 1 mg/ml. Therefore if A_{260} is equal to 1, the DNA concentration must be 50µg/ml as for example. Although this assumption is working well on purification double stranded DNA containing 50% of guanine (G) and cytosine (C), the appearance of RNA, proteins and organic solvents may allot the absorbance at the same wavelength. Therefore the use of a proportional maximum absorption of DNA (at 260nm) to protein (at 280 nm) assigned as A_{260}/A_{280} is preferable. In this experiment all pDNA possessed an A_{260}/A_{280} ratio equal or greater than 1.86.

4.2.2.2 Formulation of dendriplexes

There are three types of lysine-based dendrimers used in this study and their chemical structures are illustrated in Figure 4.2. The stock solutions of both the pDNA and the dendrimers, 10mg/ml, were prepared in sterile deionised water at the desired concentrations. The required quantity of the dendrimers and dendrons at different charge ratios (+/-) was calculated according to the following equation (Eq 4.1).

\[
\text{Amount of dendrimer/dendron (µg)} = \frac{\text{weight of DNA (µg)}}{\text{RMW of nucleotide}} \times \frac{\text{Charge ratio}}{\text{Number of charges}} \times \text{MW of dendrimer/dendron}
\]

Relative molecular weight (RMW) of nucleotide was 330. The calculated amounts of the dendrimers and dendrons (µg) were prepared and added into pDNA solution in similar volume proportion. The complexes were then swiftly mixed and incubated at room temperature, 25°C, for 30 min before being used.
Figure 4.2  Chemical structures of lysine-based dendrimers used in this work: A) a 6th generation poly-lysine dendrimer, DM1, (MW 8149 Da) containing glycine amino acid as a core and poly-lysine branches, B) a partial dendrimer (dendron) consisting of 7 lysine amino acid and eight terminal amino acid groups with a lipidic core, DM2, (MW 1758 Da) and C) DM2 dendrimer conjugated with RGD peptide, DM3, (MW 2088 Da).
4.2.2.3 Physicochemical characterisation

4.2.2.3.1 Electrophoretic retardation

The DNA binding by the dendrimer or dendron was confirmed by 0.8% agarose gel electrophoresis in Tris borate EDTA buffer (TBE buffer, 1X, see Appendix II). The gel was formed by adding 0.8 g of agarose in hot sterile water until it was fully hydrated. Ethidium bromide solution, 10mg/ml (5 μl), was added to the solution before casting. The solution was poured into a gel casting slab and the comb was inserted to create a “sample well”. The gel was left to set at room temperature for 45 min. Plasmid DNA, 2μg, and various concentrations of the dendrimer and dendron were mixed at different charge ratios (+-), 1-10 by method mentioned in section 4.2.2.2. A sample of the dendriplex, 10 μl, was mixed with the gel loading buffer (5 μl) before being immediately loaded into well. This process was carried out until all samples were loaded to achieve a final concentration of pDNA 1 μg/well. For standard, standard molecular weight marker (2 μl) was mixed with the gel loading buffer prior to application to the well. Electrophoresis was carried out at an operating voltage of 80 V for 1.5 h. The gel was then rinsed with sterile water and photograph was taken.

4.2.2.3.2 Hydrodynamic diameter assessment

Distilled water was filtered through a 0.22 μm membrane filter prior to the experiment. The dendriplexes at different charge ratios, with a final pDNA concentration of 10 μg/ml were formed in a total volume of 2 ml by simply adding dendrimer or dendron into pDNA solution as described in section 4.2.2.2. An average hydrodynamic diameter of the dendriplexes was determined at 25°C using dynamic light scattering at a fixed angle (90°) with 200 μm aperture width and 40 mW laser power. The viscosity (0.89 mPa.s) and refractive index (1.33) of distilled water at 25°C was employed for data analysis. The complexes were added into a dried cleaned cuvette. The results were analysed by CONTIN statistic. Values are provided in triplicate of the means of 10 runs of automatic measurements. This instrument was calibrated routinely before and after measurement with standard reference latex microspheres (AZ Electrophoresis Standard Kit, Malvern Instruments).
4.2.2.3.3 Zeta potential assessment

The dendriplexes were firstly prepared to achieve a pDNA concentration of 40µg/ml as recently described in 4.2.2.2. After the rapid mixing by pipetting the complexes up and down, the complex solution was diluted with sterile water to accomplish a pDNA final concentration of 10µg/ml. After 30 min incubation, the sample was injected into the capillary electrophoresis cell of a Malvern Zetasizer. The zeta potential measurement was carried out using automatic mode with a routine 5 runs with three repeat measurements.

4.2.2.3.4 Transmission electron microscopy (TEM)

Different charge ratio dendriplexes were prepared as explained in section 4.2.2.2 with a final concentration of 20 µg/ml pDNA. After 30 min incubation, a sample of dendriplexes (10-15 µl) was added to the TEM grid and the excess sample removed carefully by filter paper. The sample was then stained with 1% uranyl acetate. After the stain was dried, the grid was air-dried for 5-10 min. The photomicrographs were taken at magnifications ranging from 40,300x to 150,000x at an operating voltage ranging from 80 to 120 kV by a Philips CM 120 (Eindhoven, Netherlands).

4.2.2.4 Cell viability assessment

The cytotoxicity of the dendrimer and dendriplexes was elucidated using a colorimetric method (MTT assay) following the procedure explained by Sgouras, D. and Duncan, R. with a slight modification (Sgouras & Duncan 1990). The fact that the mitochondria of living cells are capable of converting a water soluble tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), into an insoluble formazan salt (Mosmann 1983) is exploited for quantitative measurement of mammalian cell survival and proliferation. This technique can also be applied for evaluation of biocompatibility and cytotoxicity of soluble synthetic polymers.

Caco-2 cells were seeded (1) into a 96-well plate at a density of 100,000 cells/well using a cell suspension and incubation for 3 days at 37°C in a humidified atmosphere containing 5% CO₂. Stock solutions of the dendrimer and the dendriplexes were
freshly prepared in sterile deionised water as previously described (see 4.2.2.2). The cells were then incubated, for 4 h, with the desired concentrations of the dendrimer or dendriplex (©) in a total volume of 100 μl by using fixed volume (80 μl) of DMEM as diluent under the same conditions. For a control, 20 μl of sterile deionised water was used instead of the dendrimer solution. At the end of the incubation period, the medium was aspirated from each well and the fresh medium 200 μl was added. The cells were allowed to recover in the same condition for 24 h (©).

Subsequently, the medium was removed and fresh medium 150 μl was added to each well followed by 10 μl of 5 mg/ml MTT solution (©). The cells were incubated for 2.5 h at 37 °C in dark condition, as MTT is photosensitive. Finally, the plates were inverted to remove the medium and gently tapped onto absorbent paper. DMSO, 200 μl, was added to each well and the plates were placed on a rotary shaker for 20 min to solubilise the crystals of formazan salt. An absorbance of the sample in each well was determined at 550 nm and 630 nm using plate reader machine (©), and an absorbance of the control well was read as a blank to exclude the effect of cell debris. The results were expressed as the percentage viability (n=5; ±SD). The percentage viability was calculated using the following equation. The procedures of MTT assay are summarized in Figure 4.3.

\[
\text{Cell viability (\%) = \frac{(A_{550} - A_{630}) \text{ of treated wells}}{(A_{550} - A_{630}) \text{ of untreated wells}}} \quad \text{..........(4.2)}
\]
Figure 4.3 A summary of the MTT assay. There are five major steps: ① cell seeding, ② incubation with dendriplexes, ③ cell recovery, ④ MTT incubation and ⑤ measurement of absorbance.
4.2.2.5 Quantitative and qualitative studies of transfection efficiency of the dendrimers

4.2.2.5.1 Qualitative study of transfection efficiency of dendrimers

The transfection abilities of the dendriplexes were first studied using fluorescent confocal microscopy. A plasmid DNA encoding protein that can express red fluorescent proteins was used. Caco-2 and HEK-293 were seeded on a poly-lysine coated cover slip in 6-well plates at the cell density of 1000 cells/cm² and maintained in Dulbecco's modified eagle medium (DMEM) supplement with 1% MEM non essential amino acids (MEM), 10% Foetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen/Gibco, Paisley, U.K.) at 37 °C, 5% CO₂, in 95% relative humidity until reaching 60-70% confluence. The medium in each well was changed every two days. The dendriplexes were prepared with the 10μg of DNA at the charge ratio of 2:1, 5:1 and 10:1 (+/-) as described in section 4.2.2.2. The medium was aspirated and the cells were rinsed with PBS (x2) before adding OptiMEM reduced serum medium (1 ml) into the well. The cells were next incubated with the dendriplexes for different time periods (2, 4 and 8h). Lipofectamine™ and Plus™ reagents were used as positive control and pDNA was used as negative control in the same transfecting condition as the dendriplexes.

At the end of the incubation period, the medium was removed and the cells were rinsed twice with PBS. The fresh complete medium, 3 ml, was added and the cells were maintained at 37 °C, 5% CO₂, in 95% relative humidity for 24h. After 24h the cells were rinsed with ice cold PBS twice and fixed with 4% paraformaldehyde on ice for 5 min. The cells were then washed with PBS and incubated with 50 nM ammonium chloride for 10 min to stop the action of the paraformaldehyde. The cells were washed twice with PBS before the cover slips were mounted with Citiflour® mounting media. The cell were viewed under confocal observed using 20x, 40x and 63x optical lens with single track mode; the excitation wavelength and emission wavelength used are 595 and 610 nm, respectively.
4.2.2.5.2 Quantitative study of transfection efficiency of dendrimers

The transfection efficiencies of the dendriplexes were evaluated using a luciferase assay system (Promega, USA) in SKMES/1 and HEK-293 cultures. A luciferase plasmid (7.2Kbp) was used. Both cell lines were grown in complete media (DMEM) containing 1% MEM, 10% FBS and 1% penicillin/streptomycin (Invitrogen/Gibco, U.K.) at 37 °C, 5% CO₂, in 95% relative humidity until reaching 70% confluence in 24-well plates. Complexes were formed as explained in section 4.2.2.2 with little modification by diluting 50μg of pCMV-EGFPLuc (plasmid DNA) in 125μl 4% dextrose aqueous solution. An appropriate amount of DM 1 or DM 2 was prepared in 125μl of 4% dextrose to yield complexes with 5:1, 10:1 or 20:1 (+/-) molar charge ratios. The dendrimer solution was added drop-wise to DNA solution and allowed to stabilize for 30 min at room temperature.

The complete medium was removed from each well and replaced with 500 μl of serum free medium containing dendriplexes or naked DNA at final DNA concentration of 2μg/well. Cells were incubated with the dendriplexes for 4 or 8 h at 37 °C, 5% CO₂, in 95% relative humidity. Since maximum incubation period of the cells in medium free serum should not be longer than 4h. In case of 8h incubation period, ten percent of the serum was added into the transfection medium after first 4h of incubation and cells were continue incubated for another 4 h in the same condition.

In case of the positive control the Lipofectamine™ and Plus™ reagents were used in different concentration (0.4 μg DNA/well) with 3h incubation period. Thereafter, the transfection medium was removed and replaced with the complete medium. Cells were further incubated in the same condition for 24 h before they were washed with PBS (pH 7.4), lysed with lysis buffer, and analyzed for luciferase expression using the Promega luciferase assay system kit (Promega, WI, USA). The luminescence was record using a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). Data are expressed as the mean of triplicate samples (after subtraction of the DNA only group), plus/minus the standard deviation of the mean.
4.3 Results and discussion

4.3.1 Plasmid DNA purification

The UV spectrum of the purified pDNA is demonstrated in Figure 4.4. The pDNA exhibits absorption spectrum at wavelength between 250 and 330 nm with a maximum absorption at 269 nm. The high absorbance ratios ($A_{260}/A_{280}$) of the pDNA stock solutions from two lots of DNA purification process are illustrated in Table 4.1. The $A_{260}/A_{280}$ values of the pDNA ranged from 1.86 to 1.88 which lies within acceptable limits ($A_{260}/A_{280} \geq 1.80$). The result assured that the standard method used for DNA purification is sufficient to achieve an adequate amount of pDNA with low levels of contaminant. These two lots of pDNA were used in the next experiment.

![UV/Vis spectrum of purified pDNA solution (260 nm)](image)

**Figure 4.4** UV/Vis spectrum of purified pDNA solution (260 nm): the absorbance values of the sample solutions at wavelength of 260 and 280 nm and the absorbance ratios ($A_{260}/A_{280}$) were measured and are presented in Table 6.1.

**Table 4.1** The absorbance values of the purified pDNA at 260 and 280 nm

<table>
<thead>
<tr>
<th>Sample</th>
<th>$A_{260}$</th>
<th>$A_{280}$</th>
<th>$A_{260}/A_{280}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.4439</td>
<td>0.2371</td>
<td>1.8721</td>
</tr>
<tr>
<td></td>
<td>0.4413</td>
<td>0.2371</td>
<td>1.8609</td>
</tr>
<tr>
<td></td>
<td>0.4413</td>
<td>0.2371</td>
<td>1.8609</td>
</tr>
<tr>
<td>2</td>
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<td>0.4019</td>
<td>1.8871</td>
</tr>
<tr>
<td></td>
<td>0.7530</td>
<td>0.4042</td>
<td>1.8631</td>
</tr>
<tr>
<td></td>
<td>0.7530</td>
<td>0.3997</td>
<td>1.8839</td>
</tr>
</tbody>
</table>
4.3.2 Formulation and characterization of the dendripexes

4.3.2.1 Electrophoretic retardation

Agarose gel retardation assay is a standard method for characterization and interaction between the pDNA and the carriers. The pDNA (4.7 Kb) possesses negative charge migrates to the positive polar when the electricity was applied to the system (Figure 4.5, lane 1). Complexation between the pDNA and the symmetrically cationic polylysine dendrimers or amphipathic dendrons at different molar charge ratios initiated DNA condensation and altered the charge of the pDNA as a result of electrostatic attraction between the phosphate groups of the DNA and the amino groups of the dendrimers/dendrons. Figure 4.5 demonstrates that the pDNA was condensed by both the dendrimer and dendron. At the lower molar charge ratio the dendrimer interacts with the pDNA, yet the total charge of the dendripexes remains negative (lane 3-6 in Figure 4.5) indicating insufficient amount of the dendrimer to compact the pDNA. An increase in the molar charge ratio of the dendrimers resulted in an intensification of the pDNA condensation, and the complete condensation was envisaged and scrutinized as a disappearance of the dendripexes in the gel (lane 7-8 in Figure 4.5A and lane 6-8 in Figure 4.5B). Both the dendrimers and dendrons show complete DNA condensation at molar charge ratios of 5:1 and 10:1 for symmetrical and partial polylysine dendrimer, respectively.

Interestingly, there is a difference in the condensation process between the cationic symmetric dendrimer and the amphipathic partial dendron. It was noted that there was a shift of the pDNA band obtained from the dendripexes at molar charge ratio of 1:1 (+/-) (Figure 4.5A, lane 3). Thereafter the distance of the shift of the DNA band was extended as the molar charge ratio was increased (Figure 4.5A, lane 3-8). On the contrary, this phenomenon disappeared at a similar molar charge ratio in case of the dendron. At charge ratios between 1:1 and 3:1 (+/-), the dendrons did not create a shift in DNA band until the charge ratio was increased to 4:1 and higher; the pDNA was completely condensed at 10:1 charge ratio which indicated an all or none phenomenon. This disparity might be due to the differences in their structures which are discussed as the following.
Figure 4.5  Agarose gel retardation assays of cationic dendrimer (DM1)/pDNA complexes (A) and amphipathic dendron (DM2)/pDNA complexes (B) in 0.8% agarose gel in TBE buffer demonstrates the DNA condensing capacity of the dendrimers and dendrons. The pDNA was completely condensed at molar charge ratio (+/-) of 5:1 and 10:1 for DM1 and DM2, respectively.
Two distinct models have, hitherto, been used to explained DNA condensation: a beads-on-a-string model (Widom 1998) and a DNA bundle model (Pelta, Jr. et al. 1996). The spherical shape of the dendrimers may behave as a core for the pDNA to wrap around as “beads-on-a-string” (Higashi et al. 2006). This is in accordance with the process of DNA condensation by histone proteins in Figure 4.6A (Luger et al. 1997; Bielinska et al. 1997) and other symmetrical cationic dendrimers, in Figure 4.6D (Hud & Downing 2001). The histone proteins are cylindrical and possess positive charge, thus an electrostatic attraction is seen to be the major factor in their DNA condensing capacity. For an oligo(L-lysine)-shell dendrimers (G3-PLL), an appropriate length of the lysine segment reflecting positive charge of the dendrimers has been reported as a vital parameter for DNA condensation (Higashi et al. 2006).

However, the study by synchrotron x-ray diffraction suggested that the dendriplexes were unique columnar liquid-crystalline mesophase (DNA bundles) and they were seen as flat or twisted threadlike ribbon structures in cross-polarised microscopy, Figure 4.6B-C, (Evans et al. 2003). This study was carried out using polyethyleneimine dendrimers (PEI) with diameters between 1.6-2 nm as cationic protein models. Evans and colleagues proposed that dendriplexes are columnar mesophases composed of the arrays of DNA rods intercalated with the dendrimers. Two major structures of the bundles were found: S-phase and H-phase (Figure 4.6B-C) depending on the diameter of cationic proteins and molar charge ratio (+/-) of the dendriplexes (Evans et al. 2003). H-phase (Figure 4.6B) consists of two dendrimer columns per unit cell, while S-phase (Figure 4.6C) contains only one. In the higher generation of the dendrimers, the S-phase could be maintained because an increase in steric hindrance weakening the adhesion force. On the other hand, the S-phase is likely to be stabilized by dendrimer-dendrimer repulsion force. The transition between these two phases is dependent on molar charge ratio (Evan et al. 2003). An increase in molar charge ratio of the dendriplexes after their isoelectric point (for a low generation of the dendrimer), the S-phase form can be transformed to the H-phase.

For dendrons, there are synergistic effects between the electrostatic interaction between the amino groups of the dendron and the phosphate groups of the DNA and a hydrophobic interaction between the lipidic chains of the dendrons and the DNA.
backbone. This is attributed to the interaction model proposed by Ramaswamy (Ramaswamy 2004) (Figure 4.6 E-H).

Figure 4.6 Molecular models for the molecular assembly of DNA with histone proteins (A) and dendrimers (B-H): A shows a nucleosome core particle containing the double helix of DNA (brown and turquoise) and histone proteins main chains (Blue: H3 histone, green: H4 histone, yellow: H2A histone and red: H2B histone proteins) with the perpendicular view on the right (Luger et al. 1997). B and C are the quasi-2D unit models of thread-like ribbon structures in columnar mesophase forms depending on molar charge ratio (+/-) and diameter of the dendrimers as discussed in text. The S-phase (B) and H-phase (C) models containing symmetrical cationic dendrimers (spherical particles) and the DNA (double helixes) proposed by Evans (2003). D is a “beads-on-a-string” model of the binding between the oligo(L-lysine)-shelled dendrimers (G3-PLL) and DNA (Higashi et al. 2006). E and F show the amphipathic lysine-based dendron and DNA (32bp). The dendron-DNA assembly model by Ramaswamy, C. (2004). Its top view is presented in G and H.
4.3.2.2 Particle size determination

The average hydrodynamic diameter of the dendriplexes at different molar charge ratios are presented in Figure 4.7. At low molar charge ratios, the average particle diameters of the dendriplexes are between 90.7 (dendrimer) and 107.0 nm (dendron) with the high values of polydispersity index. Since, all of the DNA could not be wrapped and condensed resulted in an appearance of various sizes of the complexes. These diameters reflect that the amount of the dendrimers and dendrons is insufficient to completely condensing the pDNA. For the dendrimers, an increase in the molar charge ratio to 2:1 (+/-) decreased the diameters of the dendriplexes, and then diameters rose again when the molar charge ratios of the dendriplexes were increased to 5:1 (+/-). An increase in the polydispersity index (PI) at a 5:1 charge ratio also confirmed the incomplete condensation of the DNA. The particle diameter of the dendriplexes was finally reduced to 85 nm at a molar charge ratio of 10:1 (+/-) suggesting a sufficient amount of dendrimer to completely condense the DNA. The increase in the molar charge ratio of the dendrimers after this did not increase the average diameter of the dendriplexes.

For the dendrons, increasing the molar charge ratio of the dendrons resulted in a decline in the size of the dendriplexes together with the reduction of the polydispersity. However, the polydispersity once more increased when the molar charge ratios of the dendrons were raised from 4:1 to 10:1, signifying that a sufficient molar charge ratio had been reached to completely condensation of the DNA was at 5:1 (+/-). An increase in the molar charge ratio of the dendron after this could result in the aggregation of the dendriplexes, as can be seen from the increase in the PI values. This agrees with the previous results obtained from the agarose gel retardation assay. The dendron created in an all or nothing fashion after the molar charge ratio reached 4:1 (+/-). The dissimilarity of the chemical structure between the dendrimer and dendrons led to clear differences in the hydrodynamic diameter of their complexes.

These results insinuated that the cationic amphipathic dendron exhibited a higher ability to condense DNA than the cationic dendrimers. This might be due to the addition of the lipidic chains in DM2 which may produce synergistic effects between
the electrostatic attraction and hydrophobic interaction between the DNA and the
dendron, while a lower hydrophobic interaction was expected in the case of DM1.

**Figure 4.7** The effect of molar charge ratios on particle diameter (brown and green columns) and polydispersity as measured by the polydispersity index (PI) (blue and orange lines) of the dendriplexes containing symmetrical poly-lysine dendrimers (DM1) and partial dendrimers (DM2) as discussed in the text. *Statistical analysis of mean diameters using ANOVA with Post Hoc test (Tukey HSD) indicates significant difference (p<0.05).

### 4.3.2.3 Zeta potential

The dendriplexes are assumed to be spheres and their surface charges are obtained from zeta potential values presented in **Figure 4.8**. The pDNA possess a negative charge. Since the dendrimer and the dendrons have positive charges, the binding between the dendrimers/dendrons and the pDNA leads to an alteration in total surface charge of the pDNA. As expected, the presence of the dendrimers and dendrons did not only regulate the particle size of the dendriplexes, but also changed the surface charge of the pDNA to positive values. An escalation of the molar charge ratio from 1:1 to 3:1 (+/-) did not significantly change the surface charge of the complexes.
implying an inadequate amount of the dendrimers to totally mask the negative charges of the pDNA. Increasing the molar charge ratios from 4:1 to 10:1 created the positive surface charge of the dendriplexes; the charge was increased when the molar charge ratio of the dendrimers was increased. In case of the dendrons, the surface charge of the dendriplexes became positive at a molar charge ratio of 2:1 (+/-). The increase in the molar charge ratio of the dendriplexes after this point resulted in the increase in the positive surface charge of the dendriplexes. The negative charge of the pDNA was completely found at molar charge ratios of 4:1 and 2:1 (+/-) for the cationic symmetric dendrimers and the amphipathic dendrons, respectively. These results reinforce the fact that the amphipathic dendrons have higher DNA condensing capacity than the dendrimers.

![Figure 4.8](image)

**Figure 4.8** The effect of molar charge ratio on the surface charge of the dendriplexes containing symmetrical poly-lysine dendrimers (DM1) and partial dendrimers (DM2)

However, different interpretations of a completely condensational point of the DNA by DM1 and DM2 were found from agarose gel retardation assay, particle size determination and zeta potential assessment. Although the result from **Figure 4.5** indicated that the DNA was completely condensed by DM1 and DM2 at 5:1 and 10:1 charge ratio, respectively, the data from particle size determination (**Figure 4.7**)
showed that the smallest particle size of the DM1 and DM2-dendriplexes could be acquired, respectively, at 10:1 and 5:1 molar charge ratio at which the DNA should be completely condensed. Whereas the result obtained from zeta potential assessment indicated that the surface charge of the dendriplexes became totally positive at 4:1 (DM1) and 2:1 (DM2) molar charge ratio (Figure 4.8). This does not correspond to the results obtained from Figure 4.5B and 4.7 as the DNA band of the DM2-dendriplexes at 2:1 ratio still remained on the agarose gel (lane 4, Figure 4.6B) in similar position to the control pDNA (lane 2) suggesting incomplete condensation of DNA. Particle diameter of the dendriplexes at 2:1 ratio (Figure 4.7) was found to be in unstable range exhibiting incomplete condensation as the increase in molar charge ratio of DM2 initiated the further decrease in dendriplex diameter reflecting the higher condensation of the DNA. Similar trend was found in DM1 (Figure 4.5A and 4.7). If we carried on examining in Figure 4.8, the zeta potential of the DM2-dendriplexes increased after the 2:1 ration and remained stable at molar charge ratio of 4:1-5:1 which may represent a real feature of complete condensation of the DNA. This may imply that two steps may involve in DNA condensing process: electrostatic interaction and condensation. The DNA may first interact with dendron by electrostatic attraction resulted in a compromise between positive and negative charge of the dendriplexes. The total charge of the dendriplexes at this step may present as positive or negative with uncertainty as shown in Figure 4.8. At the higher amount of the dendron adding to the DNA the zeta potential of the dendriplexes stable as notice from Figure 4.8 indicated that the DNA condensation was taking place (4:1-5:1(+/-)). This will be further carrified in the next section for both the DM1 and DM2-dendriplexes. To ensure that the dendriplexes are complete condensation in the next study on transfection efficiency the molar charge ratios of both DM1 and DM2-dendriplexes at $\geq 5:1(+/-)$ are recommended.

4.3.2.4 Transmission electron microscopy (TEM)

Transmission electron micrographs of the dendriplexes containing the cationic symmetric dendrimers and the amphipathic dendrons are presented in Figure 4.9 and Figure 4.10 respectively. At a molar charge ratio of 2:1 (+/-), Figure 4.9 A-B, the dendrimers are seen to be compacting pDNA into small dense particles in spherical,
oval shapes and stem-like or rod structures. Aggregation of the dendriplexes was found in every sample grids suggesting an incomplete condensation process. At the higher molar charge ratios of the dendrimer (Figure 4.9 C-H) dendrimer aggregation was still apparent and the stem-like structures of the dendriplexes were seen more dominant than the rest (Figure 4.9 C-D). The dendriplexes displayed less aggregation when the molar charge ratio of the dendrimers was 4:1 (+/-) and toroid or donut structures were observed (Figure 4.9 E-F) in the brown circular area and the insert picture). These results corresponded with the results obtained from other groups which showed that the DNA could be condensed by cationic dendrimers (Evans et al. 2003; Bielinska et al. 1997) and poly-lysine (Laemmli 1975; Lee & Huang 1996) forming compact particles with toroid and stem-like morphology. The PAMAM dendrimer was also found to initiate toroid formation with DNA and the mechanism was proposed by Dennig & Duncan (2002).

The toroidal structures were, however, less frequently seen on increasing the molar charge ratio of the dendrimers. The dense spherical structures of the complexes were abundantly observed, as can be seen in Figure 4.9 E-H. The aggregation reoccurred at a molar charge ratio of 5:1 (+/-) reflected in high hydrodynamic diameters and the high PI values of the dendriplexes discussed in 6.3.2.2. Aggregation might be due to the amount of the dendrimer which might be enough to condense the DNA, but may not be sufficient to maintain the repulsion force between particles of the dendriplexes. The dendriplexes were most homogeneous at a molar charge ratio of 10:1 (+/-) as shown in Figure 4.9 G-H. These results imply that there may be at least two steps for DNA-dendrimer assembly. The first is the spontaneous assembly between the negative charge of the phosphate backbone in the DNA and the positive amino group of the dendrimers due to the electrostatic interaction. The second is DNA condensation involving local and ultimately long-range charge neutralization (Braun et al. 2005). This second step has been claimed to be the rate-limiting step for the dendriplex formation process by Zhou (2007).

In case of the dendrons, no aggregation was observed. The dendron as surfactant properties, the presence of the lipidic chains perhaps allows hydrophobic interaction...
with the DNA molecules. As a result the dendron might bind more tightly to the DNA and condense the DNA into the spherical particles at the low molar charge ratio of 2:1 (+/-), while dendrimers do not at the same molar charge ratio. There was a sufficient amount of the dendrons to enclose the negative charge of the DNA and aggregation was hardly found (Figure 4.10 A-B). However, flocculation could be observed at low molar charge ratios. The obtained complexes of the dendron appear to be more spherical than those are found in all ranges of the dendrimers (irregular shape, oval and rod shapes). These results were in good agreement with the results of the zeta potential and hydrodynamic diameter previously obtained. The higher the molar charge ratio the smaller the particles were detected. The most homogeneous dendriplexes were found at molar charge ratio of 10:1 (+/-), Figure 4.10 C-D, indicating complete condensation of the DNA. Yet, the toroidal and stem-like structures were rarely found, illustrating the dissimilarity of the binding process between the dendrimers and dendrons and the DNA. The compositions of the hydrophilic and hydrophobic parts of the dendrons facilitate both steps of the DNA condensation and minimise aggregation.

As previously described in Figure 4.6, results from TEM, particle size determination, zeta potential evaluation and agarose gel electrophoresis suggest that a “beads-on-a-string” model may be applicable to the binding between dendron (DM2) and DNA. This can be seen in Figure 4.6 D, H and G. In case of the DM1, the spherical shape of the dendrimer might be explained by both a “beads-on-a-string” model and a columnar mesophase model depending on the molar charge ratios of the complexes. At lower molar charge ratios, the complexes frequently found in a rod shape indicated that the intercalation between DNA and DM1 is in a more columnar structure as illustrated in Figure 4.6 B-C and Figure 4.9 A-F. At higher molar charge ratios this rod shape disappears suggesting the bead on string model may take precedence.
Note: see the caption in the next page*
Figure 4.9 Transmission electron micrographs present different stages of DNA condensation at various molar charge ratios (+/-) of the 6th generation poly-lysine dendrimer (DM1) including 2:1 (A-B), 3:1 (C-D), 4:1 (E-F), 5:1 (G-H) and 10:1 (I-J). The dendriples were formed in sterile water by adding the dendrimer solution into the DNA solution at a similar volume ratio. After swift mixing, the complexes were left at room temperature for 30 mins prior to visualisation. DNA is seen to be compacted by the dendrimer into small dense particles in spherical, oval shapes and stem-like or rod structures. Aggregation of the dendriples was noticed in all sample grids at low molar charge ratios (A-D), indicating an incomplete condensation process. The dendriples display less aggregation at higher molar charge ratios (E-J) and at 4:1(E-F) charge ratio toroidal structures are observed. Aggregation is, however, reappearing at 5:1 (G-H) before disappearing at 10:1(I-J) suggesting a complete condensation.
Figure 4.10  Transmission electron micrographs display the DNA condensation process at two molar charge ratios (+/-) of the partial poly-lysine dendrimer (DM2): 2:1 (A-B) and 10:1 (C-D). The homogeneous size of the complexes is obtained at molar charge ratios of ≥ 2:1 corresponds to the hydrodynamic diameter acquired from photon correlation spectroscopy shown in Figure 4.7.
4.3.3 Cytotoxicity assessment (MTT assay)

Cytotoxicity results for the cationic symmetric dendrimer in four different cell lines are illustrated in Figure 4.11. Human colon adenocarcinoma cell line (Caco-2) is regularly used as a standard model of human intestinal epithelium cell for absorption and permeability studies (D'Emanuele et al. 2004). Human embryonic kidney cell line (HEK-293), human Caucasian lung squamous carcinoma cell line (SKMES-1) and rat embryonic fibroblast cell line (REFS-2) are commonly used for transfection studies (Choi et al. 2006). Although the cell lines were exposed to the dendrimers for 4h, the dendrimers were found to be toxic in all cell lines, the inhibitory concentrations (IC$_{50}$) of the dendrimers were 7.5, 4.5, 3.5 and 6.5 mg/ml in Caco-2, Hek-293, SKMES-1 and REFS-2, respectively. The discrepancy in the IC$_{50}$ indicated the maximum amount of the dendrimers that could be used in each cell line in the transfection study.

In the Caco-2 cell line, at low concentrations of the dendrimer, 0.25-2.5 mg/ml, the viability of the cells remained stable at between 89-91% before reducing to 83% at a concentration of 5 mg/ml and it finally levelled off at 12% at 10 mg/ml of the dendrimers as seen in Figure 4.11A. This result is in good agreement with the results obtained by Al-Jamal, K.T. (2006). The dendrimers exhibited more toxicity in HEK-293 cells (Figure 4.11B). The viability initially fell from 94-90% at dendrimer concentrations of 0.25-1 mg/ml, and decreased to 63, 48 and end at 27% with the dendrimer concentrations of 2.5, 5 and 10 mg/ml, respectively. An increase in toxicity was found in SKMES-2 cells. Viability (%) lies between 80-105% at dendrimer concentrations between 0.25-2.5 mg/ml prior to a steep decrease to 15 and 13% at a dendrimer concentration of 5 and 10 mg/ml, respectively (Figure 4.11C). A similar trend found in Caco-2 cells was found in REFS-2 cells. The viability initiated at 91% at a dendrimer concentration of 0.25 mg/ml declined to 84, 83, 76 and 64% at dendrimer concentrations of 0.5, 1, 2.5 and 5 mg/ml, respectively, before sharply falling to 18% at a dendrimer concentration of 10 mg/ml. These results were in accordance with the results obtained by other groups (Malik et al. 2000; Duncan & Izzo 2005)
Figure 4.11  Cytotoxicity of the 6th generation poly-lysine dendrimer (DM1) in Caco-2 (A), HEK-293 (B), SKMES-1 (C) and REFS-2 cultures (D): the cells at 70-80% cell confluence were exposed to various concentrations of the dendrimers for 4 h and allowed to recover for 24 h before measuring cell viability.

The cytotoxicity of dendriplexes, containing 1 µg of pDNA/well (10 µg/ml), was studied in comparison to that of naked DNA (1 µg/100 µl/well or 10 µg/ml) and the dendrimer per se (3.86 µg of DM1/100 µl/well or 38.6 µg/ml) in Caco-2 and HEK-293 cells, as can be seen in Figure 4.12. The highest concentration of the dendrimers
(DM1), 3.86 μg/well, used to form complexes with pDNA (1μg) at a molar charge ratio of 10:1 (+/-) was used as a control. Cells were exposed to the dendriplexes or pDNA or the dendrimers for 8h before their viability were elucidated. As expected, the naked DNA and dendrimer (DM1) exhibit slight toxicity in both types of cell lines. Percent viability of cells exposed to naked DNA and DM1 was found to be 94.1 and 94.98 % in Caco-2, and 92.3 and 96.4 % in HEK-293 cells, respectively.

Although the dendrimer exhibited cytotoxicity in four types of cell lines as shown in Figure 4.11, the dendrimer at concentrations required to form complexes with pDNA at various molar charge ratios (1:1-10:1(+/−)) was found to have a much lower toxic effect. Results in Figure 4.12 demonstrate that all concentrations of the dendrimer and the dendriplexes used in transfection study show very low toxicity in both types of cell lines. Statistical analysis of mean cytotoxicity using one-way ANOVA indicated significantly different of the dendriplex at 5:1 charge ratio among all concentrations of pDNA, dendrimer and dendriplexes used (p<0.05) in Caco-2 cells. The percent viability of cells exposed to the dendriplexes at molar charge ratios of 1:1, 1:2, 5:1 and 10:1 (+/-) was, respectively, found to be 95.5, 96.5, 111.2 and 101.8 %. Those of HEK-293 exposed to the dendriplexes at the same molar charge ratio was found to be 92.2, 93.5, 101.4 and 94.2 %, respectively. It is well known that the cytotoxicity of polycationic polymers can be minimized by their conjugation with other molecules and anionic molecules (Choi et al. 2006; Twaites et al. 2005). Similarly, in this study we found that the dendrimer (DM1) can provide a dendriplex at high molar charge ratio (10:1, +/-) with insignificant toxicity.

Cytotoxicity of the dendriplexes containing amphipathic asymmetric poly-lysine dendrimers (DM2) were well established (Ramaswamy 2004) and the dendriplexes could therefore readily used regard to that condition. The optimum incubation period for transfection was 4-6h at DNA concentrations of 1-20 μg/ml.
Cytotoxicity of the dendriplexes containing 6th generation poly-lysine dendrimers (DM1) in Caco-2 and HEK-293 cells: the cells at 70-80% cell confluence were exposed to the dendriplexes at different molar charge ratios for 8 h and allowed to recover for 24 h prior to measuring percentage of cell viability. Plasmid DNA and the DM1 were used as control. *Statistical analysis of means of % viabilities using ANOVA with Post Hoc test (Tukey HSD) indicated significant difference (p<0.05).

4.3.4 Transfection studies

4.3.4.1 Qualitative study of transfection efficiency of dendrimers

The transfection ability of the dendrimers (DM1) in Caco-2 and HEK-293 cells is illustrated in Figure 4.13 and 4.14. Small amounts of cells were transfected by naked DNA (Figure 4.13 B-C). Transfection ability of the dendrimers depends on incubation period and molar charge ratio. The higher the molar charge ratios the greater transfection (Figure 4.13 E-J). An incubation period of 8h is optimal for this type of the dendrimer (Figure 4.13 E-J). A similar trend was observed in HEK-293 cells as can be seen in Figure 4.14. Dendrimers facilitated transgene expression in both types of cells in comparison to naked DNA.
T=4h

T=8h

T=4h

T=8h

T=8h

Note: see the caption in the next page*
Figure 4.13 Transfection ability of the symmetrically cationic poly-lysine dendrimers (DM1) at different molar charge ratios (+/-): 2:1 (E-F), 5:1 (G-H) and 10:1 (I-J) in Caco-2 cells, pDNA encoding a red fluorescent protein (C-D). Dendrimers (A-B) were used as a control. The cells were incubated with the pDNA and dendriplexes for 4 and 8 h in Optimem-I reduced serum media before rinsing with PBS and incubating for a further 24h. The cells were then fixed with 4% paraformaldehyde and visualised under confocal microscopy. The protein expression is displayed in by the red colour. Transfection ability depends on molar charge ratio and incubation period. The results indicated that strong gene expression is acquired at the 10:1 charge ratio of the dendriplexes with an incubation period of 8h (as see in I and J).
Figure 4.14 Transfection ability of the symmetrically cationic poly-lysine dendrimers (DM1) at different molar charge ratios (+/-): 5:1 (C-D) and 10:1 (E-F) in HEK 293 cells in comparison to pDNA per se (A-B). The figures (C-F) indicated the effects of molar charge ratio and incubation period on transfection efficiency of DM1 and DM2. The complexes at 10:1 charge ratio (+/-) (E-F) shows higher transfection than that at 5:1 charge ratio (C-D) at both incubation periods (4 and 8h).
Figure 4.15  Transfection abilities of the amphipathic dendrons, DM2 (C) and amphipathic dendrimers with RGD peptide conjugated, DM3 (D) in HEK-293 cells: pDNA encoding a red fluorescent protein (A) and Lipofectamine™ and Plus™ reagents, LPP, (B) were used as negative and positive controls, respectively. The cells were incubated in reduced serum media with the pDNA and dendriplexes, 10:1 (+/-), for 4 h, before rinsing with PBS and incubating for a further 24h in the same condition. The cells were fixed with 4% paraformaldehyde and counter stained the cell nuclei with DAPI (blue channel, Ch2) prior to be visualised under confocal microscopy. The protein expression is displayed in red channel (Ch1). Ch3 is taken with transmission light (trans). The complexes of DM2 and DM3 show higher gene expression (see here as red fluorescent signal) than that of LPP reagents and naked DNA.
In case of the amphipathic branched-lysine dendrons, the existence of the lipidic chains may confer advantages in terms of their ability to assemble and disassemble with DNA and eventually enhance gene transfection. Figure 4.15 exemplifies the transfection results of the amphipathic poly-lysine dendrons (DM2) and the amphipathic poly-lysine dendrons with RGD-peptide conjugated (DM3) in a comparative study with naked DNA (pDNA) and Lipofectamine™ together with Plus™ reagents (LPP). The dendron exhibited higher gene transfer as can be seen in Figure 4.15A-C. There was no gene expression observed by naked DNA (pDNA) (Figure 4.15A). The results may be attributed to the small size of the dendriplexes, 60-80 nm in diameter, which facilitate the uptake of the complexes into the cells by endocytosis. This small size was caused by the presence of the lipidic chains which confers the utility of both hydrophilic and hydrophobic interactions with DNA. The hydrophobic moieties may be involved in the uptake, endosomal escape and DNA disassembly processes. The explanation is still a mystery. The dynamics of uptake of the complexes is discussed in the next chapter.

Arginine-glycine-aspartate sequences, known as RGD peptides, have been found to promote cell adhesion due to their specific interaction with integrins, the glycoproteins found on the cell surface (Dunehoo et al. 2006). This peptide sequence has been employed in various applications including drug targeting, drug design and radiolabeling (Meyer et al. 2006). The conjugation of dendrons to RGD peptides could, therefore, improve transgene ability of the dendrons as can be seen in Figure 4.15C-D. There was a broad spreading of the red fluorescent protein expressed inside almost of the cells and the fluorescent signal was found to be stronger than that found in cells transfected with dendriplexes without the conjugating peptide. Poly-lysine and other cationic polymers have been proved to have the ability to condense DNA, but linear poly-lysine lacked a lysomotrophic effect, which reduces its transfection efficiency (Haensler & Szoka, Jr. 1993). To date, there is also no evidence on this effect for our lysine dendrimers. The transfection results of the polycationic poly-lysine dendrimers (DM1) shown in Figure 4.14 suggest a lack of lysomotrophic effect in the cationic symmetric lysine dendrimer (DM1) as only a minority of transfected cells could be detected. On the contrary, the hydrophobic head groups in dendrons
DM2 and DM3 not only intensified the DNA condensation of the dendriplexes but also enhanced transfection in target cells. Either an ability to escape from endosome or a lysomotropic effect is expected to be found in DM2 and DM3. To be able to explain this, further studies are indispensable.

4.3.4.2 Quantitative study of the transfection efficiency of dendrimers

Figure 4.16 demonstrates the transfection studies of dendriplexes of DM1 (A-B) and DM2 (C-D) at 5:1, 10:1 and 20:1 molar charge ratios (+/-) in SKMES-1 (A and C) and HEK-293 cells (B and D) using the luciferase assay. Three main parameters to achieve efficient transfection were evaluated: molar charge ratios of the dendriplexes (5:1, 10:1 and 20:1 (+/-)), incubation periods (4 and 8h) and types of cell lines. Naked DNA and lipofectamine™ together with plus™ reagents (Lipofectamine-Plus™ (LPP)) were used as negative and positive controls, respectively. Generally, HEK-293 cells are likely to get better transfection than SKMES-1 cells for both types of dendrimers (Figure 4.16A and D). At maximum transfection efficacy, DM2 shows higher transfection than DM1 (Figure 4.16 B and D). The longer the incubation period the higher transfection is observed (Figure 4.16 A-D). An increase in molar charge ratios of the dendriplexes resulted in an increase in transfection efficiency.

DM1-dendriplexes at 5:1 (+/-) display lower transfection efficiencies than that of naked DNA (Figure 4.16 A) in SKMES-1 cell. The opposite was found in HEK-293 cells at the same molar charge ratio (Figure 4.16 B). At the higher molar charge ratios the dendriplexes exhibits higher transfection than naked DNA in both cell lines. Therefore the optimal condition of the DM1 dendrimer is at 20:1 charge ratio of the dendriplexes for 4 h incubation period. By comparison with DM2-dendriplexes in the same cell lines, the DM2-dendriplexes at 10:1 (+/-) showed significantly (p<0.05) higher transfection efficiency than the DM1- and DM2-dendriplex at other molar charge ratios. The statistical analysis of mean transfection efficiency was carried out using one-way ANOVA. This is in good agreement with the results obtained by Ramaswamy (2004) suggested that the lipidic chained of the dendrimer may improve DNA condensation via hydrophobic interaction, and this lipidic tails could perhaps
play a decisive role in uptake pathway and endosomes escape of the dendriplexes. Future works are needed.

Figure 4.16 Quantitative transfection study of DM1 and DM2 dendrimers illustrates thee parameters impinge on proficient transfection of the dendriplexes: molar charge ratio, incubation period and type of cell lines. The transfection studies of dendriplexes of DM1 (A-B) and DM2(C-D) at 5:1, 10:1 and 20:1 molar charge ratios (+/-) were carried out in SKMES-1 (A and C) and HEK-293 cells (B and D) using the luciferase assay. HEK-293 cells appear to be more amenable to transfection than SKMES-1 cells for both types of dendrimers (A and D). At maximum transfection efficacy, DM2 shows higher transfection than DM1 (B and D). The longer the incubation period the higher transfection is observed (A-D). The optimal transfection conditions for DM1 and DM2 are, respectively, at 20:1 and 10:1 for 4 h of incubation period. *Statistical analysis of mean RLU/well using ANOVA with Post Hoc test (Tukey HSD) indicated significant difference of the transfection efficiency of the DM-2-dendriplexe at 10:1 molar charge ratio (p<0.05).
For the DM2 dendrimer, the dendriplexes at all molar charge ratios exerts higher transfection efficacy than that of the naked DNA as can be seen in Figure 4.16 C-D. A similar trend observed in DM1 is also found here. The increase of molar charge ratios and incubation period enhanced the transfection in both cell lines. However, the transfection was found to reduce at the 20:1 molar charge ratio for both incubation periods, in HEK-293 cells indicating there was saturation for transfection of the dendriplexes in this cell type (Figure 4.16D). This might be due to toxicity effect of the dendriplexes. As the MTT study did not cover the toxicity of the dendriplexes at 20:1 molar charge ratio; further study on this has to be carried out. The RLU unit/well of the LPP was found to be $0.91 \times 10^8$ (SD, $\pm 0.06 \times 10^8$) and $0.78 \times 10^8$ (SD, $\pm 0.02 \times 10^8$), respectively, in SKMES-1 and HEK-293 after 3 h instead of 4h of incubation which was performed with the dendrimer/dendron. Since the conditions used for the LPP are different in both concentrations (0.4 µg of DNA/well) and incubation period (3h) from those which obtained in Figure 4.16, the data therefore can not be directly compared. Overall, the optimal transfection condition of DM1 and DM2 are, respectively, at 20:1 and 10:1 for 4 h of incubation period.

4.4 Conclusion

A cationic symmetric poly-lysine dendrimer (DM1) and an amphipathic asymmetric poly-lysine dendrimer, DM2, have the ability to condense DNA due to their electrostatic interaction between the positive charge of their amino groups and the negative charge of the phosphate groups in the DNA backbone. There are at least two processes for dendrimer-DNA assembly: 1) simultaneous assembly emerged between the amino groups of the dendrimers and the phosphate groups of the DNA backbone and 2) the process of DNA condensation initiated by local and ultimate charge neutralization inside the dendriplexes. The DNA condensation depends on dendrimer structure, molar charge ratio (+/-) and concentration of salt in the medium. The existence of the lipidic chains allowed a synergistic effect between electrostatic and hydrophobic interactions, and this is confirmed by the results obtained in agarose gel electrophoresis. DM2 could achieve complete DNA condensation with a positive surface charge and smaller size at a lower molar charge ratio than DM1.
Key properties of the dendriplexes include particle size, shape and surface charge are important parameters to accomplish high gene transfer. DM1 and DM2 were capable of delivering DNA but the transfecting ability of the dendrimers was related to incubation duration, cell types and the molar charge ratio of the dendriplexes. Transfection efficiency of the DM1 and DM2 were qualitative and quantitative studied and the results suggested the optimum molar charge ratio at 20:1 and 10:1(+/−) for both DM1 and DM2 at an optimum incubation period of 4h, respectively. At maximum transfection both types of dendrimers displayed 1,000 fold (DM1) and 10,000 fold (DM2) higher transfection efficiency than that of naked DNA (Figure 4.16) respectively, suggesting these dendrimers may be used as transfecting agents. Since the conditions used for transfection of the LPP using the luciferase assay are different from DM1 and DM2 the data can not be directly compared. However the qualitative results from confocal microscopy (Figure 4.15) which all LPP (Lipofectamine-Plus\textsuperscript{TM}), DM2 and DM3 (RGD-conjugated DM2) were treated with the same transfecting conditions (at concentration of 10μg DNA/well and 4h incubation period) have revealed that the transfection efficiency may be found in ascending order: pDNA<DM1<LPP<DM2<DM3. The further quantitative study is needed to confirm this result.

In fact, one may argue about this conclusion as the data obtained here are qualitative data and the structure of the dendrimer and the dendron are totally different. The point of this study only intends to explore the new application of the DM1 dendrimer and one possibility should be as a transfecting agent. To investigate this possibility the reference standard of the dendrimer containing a similar branch unit is indispensable. However, the transfecting agent of lysine-based dendrimer is not commercial available. The DM2 expected to be the best transfecting agent available in our group and thus it was selected for this purpose.
Chapter V

DYNAMIC UPTAKE OF AN INTRINSICALLY FLUORESCENT DENDRIMER AND DENDRIPLEXES

Summary

Macromolecular crowding and the presence of organelles in the cytosol present barriers to particle mobility, such that it is unclear how nanocarriers can deliver their active agents to the nucleus. In this chapter a sixth generation amino terminated polyamide polylysine dendrimer, \((\text{Gly})_{63}(\text{Lys})_{64}(\text{NH}_2)_{64}\) (MW 8149 Da, diameter 6.5 nm), which is fluorescent allowed the study of nuclear uptake and mobility in living lung carcinoma (SKMES-1) and colon adenocarcinoma (Caco-2) cells. The dendrimer is found within 25–55 min of incubation inside the cell nuclei. Living cells were then used to develop a method for dynamic nuclear uptake study using confocal microscopy. The dynamic uptake of the dendrimer demonstrated here allowed the apparent cytoplasmic diffusion coefficient \((D_{cyto})\) of the dendrimer and the dendriplexes to be calculated. Values were found in the range \(5.99 \times 10^{-11} \text{ cm}^2 \text{ s}^{-1}\) (in SK/MES-1 cells) to \(9.82 \times 10^{-11} \text{ cm}^2 \text{ s}^{-1}\) (in Caco-2 cells) for the dendrimer and \(2.36 \times 10^{-11} \text{ cm}^2 \text{ s}^{-1}\) (in Caco-2 cells) for the dendriplexes. The difference must reflect variation in the intracellular architecture of the cell types and in effect of the particle size which are discussed here.
5.1 Introduction

A comprehensive knowledge of uptake and the translocation of dendrimers within cells are undoubtedly crucial for the design and development of effective gene carrier systems. However, dendrimer uptake by cells has, hitherto, been studied by exploiting the attachment of fluorescent probes onto dendrimers either by chemical conjugation (Jevprasesphant et al. 2004) or physical interaction (El-Sayed et al. 2001); hence the degradation of the probe from the parent dendrimer throughout experimentation cannot be guaranteed. This may result in difficulty in interpretation of data. The use of an intrinsic fluorescent dendrimer can overcome these problems. Firstly, there is no need for a fluorescent labelling; the processes of purification and characterisation of compounds after the conjugation/labelling can be abridged. Secondly, the precision in the dynamic feature of the dendrimer can be accurately determined as the mobility of the parent dendrimer would otherwise be risk of the contamination of free probes. Lastly, the detachment of the fluorescent probe from the parent dendrimer during the uptake and transport process is avoided. In fact, an incidence of the uptake of the unreacted low-molecular weighted fluorescent probe may appear as endocytosis of the conjugated molecule, as reported by Preston (Preston et al. 1987).

We recently reported a sixth generation amino terminated polyamide poly-lysine dendrimer – namely (Gly)(Lys$_{63}$(NH$_2$)$_{64}$ with a molecular weight 8149 Da – as a “nano-fluorescent probe” (Al-Jamal et al. 2006). The potential use of this compound as a nano-probe for its dynamic uptake mechanism into cell cytoplasmic and nuclear compartments is discussed in this chapter using a direct method with confocal laser scanning microscopy (CLSM). The results of the uptake and mobility of this nano-probe in cells are discussed in terms of dendrimer exclusion, obstruction and binding effects within the cytoplasm. Some relevant terminologies have to be addressed here.

5.1.1 Dendrimer uptake

To date, as has been discussed in Chapter I, dendrimers have been employed in various medical applications encompassing diagnostic tools, drug carriers and as gene carrier systems. Despite these numerous applications, elucidation of the uptake
mechanisms and the cellular pathways of dendrimer carrier systems and dendrimers per se have been relatively slow. The majority of the literature claims that dendrimers are taken up mainly by endocytosis (Haensler & Szoka, Jr. 1993; Manunta et al. 2004; Najlah et al. 2007; Paleos et al. 2007; Jevprasesphant et al. 2003; Manunta et al. 2006). A similar uptake mechanism has also been found in cationic lipid (Figure 5.1A) and polymeric carrier systems (Figure 5.1B) (Elouahabi & Ruysschaert 2005). Some research groups have, however, reported that some dendrimers may create nano-holes ranging between 15-40 nm in cell membranes allowing passive diffusion of the dendriplexes (Figure 5.1C) (Hong et al. 2004; Hong et al. 2006; Mecke et al. 2005c; Mecke et al. 2005a; Mecke et al. 2005b). In addition, polylysine polymer was found to initiate nano-holes on cell membrane with a diameter ranging from 1 to 10 nm (Hong et al. 2006).

A summary of uptake mechanisms and cellular pathways of cationic lipoplexes and dendriplexes-mediated transfection is illustrated in Figure 5.1. Both lipoplexes and dendriplexes possessing positive charges first adhere to the cell membrane as a result of electrostatic interactions (1). Mislick and Baldeschwieler (1996) demonstrated that membrane-associated proteoglycans serve as a receptor for transfection by dendriplexes and lipoplexes. Endocytosis has been invoked depending on types of carrier systems and cell lines (Mislick & Baldeschwieler 1996; Manunta et al. 2007). The constituents of the carrier surface, in fact, regulate specific or non-specific binding of the complexes onto the cell membrane and trigger different sub-endocytosis pathways such as clathrin- and caveolin-mediated endocytosis (1A and 1B), clathrin-and caveolin-independent endocytosis (1D and 1E)). Clathrin- (1A) and caveolin-mediated endocytosis (1B) has been proposed as the major pathway of internalization of lipoplexes and dendriplexes (Woodle & Scaria 2001; Manunta et al. 2006; Elouahabi & Ruysschaert 2005).
Dendrimer-based polymers

Figure 5.1 A summation of putative uptake mechanisms and cellular pathways of cationic lipoplex- and dendriplex-mediated transfection. Lipoplexes and dendriplexes are depicted as spheres of green (cationic lipid)/blue (cationic dendrimer) and brown colours (plasmid DNA). First, both lipoplexes and dendriplexes possessing positive charges adhere to the cell membrane due to electrostatic interaction (1). Endocytosis has been established depending on type of carriers and cell lines; constituents in the carrier surface indicated specific or non-specific bound on cell membrane initiating a subtype of endocytosis pathway (clathrin- and caveolin-mediated endocytosis (1A and 1B), clathrin-and caveolin-independent endocytosis (1D and 1E)). Clathrin- (1A) and caveolin-mediated endocytosis (1B) has been proposed as mainly internalised pathways of lipoplexes and dendriplexes, respectively. However, some research groups have provided evidence of nano-hole formation, 15-40 nm in diameter, in the cell membrane allowing passive diffusion of the dendriplexes (1C). The endosomes are then transported to the perinuclear region where the released DNA would have a greater chance to enter the nucleus (1). Escaping from endosomes plays an active role in discrimination between lipoplexes (1, 2) and dendriplexes (3). The hydrophobic interaction initiates membrane fusion (4) and membrane destabilization (5) and plays a critical role in endosome escape of lipoplexes (Xu & Szoka, 1996; Elouahabi & Ruyschaert 2005). Hydrophilic dendriplexes bearing tertiary amines, employ other mechanisms including the so called the “sponge effect” (6, Kichler et al. 2001; Kamiya et al. 2001; Tang et al. 1996; Kukowska-Latallo et al. 1996; Boussif et al. 1995). Dissociation of the lipoplexes and dendriplexes from endosomes may result in the release of plasmid DNA from the carriers (1, 2) and some of the plasmid DNA/dendriplexes/lipoplexes may interact with cellular organelles and macromolecules. Lipoplexes and dendriplexes incapable of escaping from endosomes are liable to be degraded by the lysosomes (6). Finally the released plasmids enter nucleus either by passive or active transport as explained in text.
5.1.2 Nuclear uptake

The cell nucleus is the ultimate target to achieve efficient gene transfection. Nevertheless the exact process of how dendriplexes delivery their plasmid DNA to nuclei after escaping from endosomes has remained elusive (©). The nuclear pore complex (NPC) responsible for nucleocytoplasmic transport - the traffic of matter between nucleus and cytoplasm (Allen et al. 2000; Rout et al. 2000) - plays a fundamental role in human and other eukaryotic cells, affecting almost every aspect of health and disease. The NPC possesses remarkable transport competencies with two distinct modes: passive and facilitated (or active) transports (Lusk et al. 2007; Bickel & Bruinsma 2002). Passive transport is a nonspecific process which involves ordinary diffusion of matter through the nuclear pore with a cutoff at about 10 nm in diameter (Peters 2006) and/or molecular mass less than 25 kDa (Lusk et al. 2007). Facilitated transport is the highly specific process acting against concentration gradients (Dingwall et al. 1982) with cut-off diameters at approximately 50 nm (Peter, R., 2006) and/or molecular mass (m) of 25<m<75 kDa (Lusk et al. 2007). This limit easily includes the size of most protein transport substrates and macromolecules, for which the molecular weight of the cargo-receptor complex should be in the low hundreds of kDa.

In gene therapy, transport of plasmid DNA to the nucleus (©) is also a biphasic processes. For passive mode, the DNA is likely to enter the nucleus by passive diffusion during mitosis when the nuclear membrane is broken down. Therefore the cell division activity and plasmid stability are key factors to achieve passive transport pathway. However the passive diffusion of the DNA through nuclei in non-mitosis cells has been reported (Salman et al. 2001; de Gennes 1999). Salman et al. (2001) found that the uptake of DNA, 2nm in linear diameter, is independent of ATP or GTP hydrolysis indicating linear diffusion without the need for conformation change or specific biochemical interaction with nuclear pore complexes (NPC). Salmon suggested that DNA was passively sliding through nuclear pore from one end to another chain end of its λ-phase form. The kinetic results, however, show diffusion to be much slower than would be estimated from purely hydrodynamic considerations. This, perhaps, suggested that the DNA may possible disassembly from its carrier
before it passively diffuses through NPC into the nucleus. Once through the NPC the journey of the DNA is not over. The nature of the nucleus is crowded. Active transport entails nuclear localisation signal (NLS) peptides which can be linked to the plasmid DNA; this peptide triggers the transport of the DNA through NPC by forming a complex with nuclear membrane importers.

So far, less attention has been paid to this nuclear uptake process and none of conclusion has been drawn. The solution of this puzzle will improve understanding to aid the rational design of effective vector for gene therapy.

5.1.3 Diffusion within cells

Unlike dilute or bulk solutions cell interiors are rather more concentrated and crowded as they are occupied by a variety of macromolecules between 5% and 40% of the total cell volume or 400 mg/liter (Ellis & Minton 2003). The cellular architecture provides a complex non-Newtonian fluid comprising an aqueous phase filling the spaces between an entangled mesh of filamentous cytoskeleton and other macromolecular structures resembling a gel-like structure (Fulton 1982; Goodsell 1991; Luby-Phelps 2000; Medalia et al. 2002). This phenomenon is well known as “macromolecular crowding” which plays a decisive role on several levels of cellular organisation particularly in the diffusion and binding of matter inside cells (Luby-Phelps 2000).

As explained in Chapter III, diffusivity of solute in water or bulk solution depends mainly on the temperature and the size of the solutes as predicted by the Stokes-Einstein equation, which may not be applicable to diffusion phenomenon within cells. Diffusion of matter in cells relies on fluid-phase viscosity (F₁), solute binding to macromolecules (F₂) and collisions between solutes and macromolecules (F₃) (Zimmerman & Minton 1993; Kao et al. 1993). Diffusion theory of the solutes in the cell cytoplasm was mathematically described by Kao et al. (1993). They assumed that cell cytoplasm is composed of an aqueous fluid-phase compartment bathing a matrix of mobile and static macromolecules/particles that are much larger than the water molecules and small solutes. Three main factors are involved in the reduction in
diffusion coefficient of a small solute in cytoplasm (D_{cyto}) relative to that in water (D_0). These factors can be defined as below where

\[
\frac{D_{cyto}}{D_0} = F_1(\eta) \times F_2(D_u, \{D_{b,i}, f_{b,i}\}) \times F_3(\{n_i, V_i\}) \quad \ldots (5.1)
\]

The function \(F_1(\eta)\) represents the deceleration of net solute translational diffusion because of an increase in true fluid-phase cytoplasmic viscosity. This increase would display some solute-induced perturbation in solvent structure, which need not be specified. This can be written as in equation 5.2.

\[
F_1(\eta) = \frac{\eta_0}{\eta_{cyto}} \quad \ldots (5.2)
\]

Where \(\eta_0\) and \(\eta_{cyto}\) represent, respectively, the viscosities of water and the true fluid-phase microviscosity of cell cytoplasm.

The function \(F_2(D_u, \{D_{b,i}, f_{b,i}\})\) indicates the total reduction of solute translational diffusion regarding the transient binding of solute molecules to cytoplasmic structures as addressed in equation 5.3 (Kao et al. 1993). The multiple bound species can be evaluated; \(F_2\) represents the ratio of the weighted diffusion coefficient of bound and unbound solute to the diffusion coefficient of the unbound solute. Whereas \(D_u\) and \(D_{b,i}\) are, respectively, the diffusion coefficients of unbound and \(i\)th bound solute, and \(f_{b,i}\) is the fraction of net solute bound to component \(i\).

\[
F_2(D_u, \{D_{b,i}, f_{b,i}\}) = f_u + \sum_i (D_{b,i}/D_u) f_{b,i} \quad \ldots (5.3)
\]

Where

\[
f_u + \sum_i f_{b,i} = 1 \quad \ldots (5.5)
\]

The function \(F_3(\{n_i, V_i\})\) illustrates the deceleration of the total reduction of solute translational diffusion owing to collisional interaction with cytoplasmic structures assuming there are \(n_i\) structures of type \(i\), each possess volume \(V_i\). The "volume exclusion" by mobile obstacles on diffusion has been used as model (a stretched-exponential (SE) model) to explain this function (Furukawa et al. 1991; Phillips 1989; Hou et al. 1990). This SE model is applicable for the diffusion of Brownian particles (solute) which are relatively larger than the solvent size.
\[ F_{1,5}(n_i, V_i) = \exp[-\alpha(n_i V_i)^\gamma] \]  

where \( n_i V_i \) represents the volume fraction occupied by the occluding molecule and the prefactor \( \alpha \) and \( \gamma \) represent scaling parameters obtained by fitting Eq. 5.5 to data and can be calculated independently from theory (Kao et al. 1993).

Viscosities of the cytoplasm of various cell types have been calculated and ranged from 2-20 cP (Clegg 1984; Mastro et al. 1984; Nicolay et al. 1995; Kao et al. 1993). By using time resolved fluorescence anisotropy, Verkman and colleagues found that the cytoplasmic solvent measured in different types of cell lines may be significantly different from that of bulk water (Verkman et al. 1991). Similarly, the same aspect was also demonstrated by Luby-Phelps (Luby-Phelps et al. 1993) by a different approach in two mammalian tissue culture cell lines. Therefore, the reduction of the translational diffusion of particles or molecules in the cytoplasm should be attributed to microscopic barriers. This corresponds to the decrease in diffusion coefficient (\( D_{cyto} \)) of fluorescent probe in cytoplasm by comparison with that in water or dilute solutions; such an effect is underlined with larger molecules (Luby-Phelps et al. 1987; Luby-Phelps & Taylor 1988; Luby-Phelps et al. 1986).

Luby-Phelps et al. (1987) characterised the nature of the diffusion barrier by comparing the diffusion of 3.2-25.8 nm Ficoll probe molecules. The results show that the relative diffusion rate in cytoplasm, which is already low with the 3.2 nm probe, reduces with an increase of the probe size as shown in Figure 5.2A. To describe this relationship, diffusion of various sizes of FITC-Ficoll was measured in model solutions (Hou et al. 1990). As can be seen from Figure 5.2B the relative diffusion coefficient of FITC-Ficoll in concentrated solutions of Ficoll is low, but it is independent of the size of the FITC-Ficoll probe. In a tangle of F-actin filaments, on the other hand, relative diffusion of the 3.2 nm probe is not impeded, but it decreases with the larger size of FITC-Ficoll probes (Figure 5.2B). Taken together, it was noticed that the effects of Ficoll (10%) and F-actin filaments (5mg/ml) resemble the pattern seen in cytoplasm. The solution conditions calculated to most closely mimic the size dependency of FITC-Ficoll diffusion in the cells are 120 mg/ml of bovine serum albumin dissolved in a tangle containing 37 mg/ml F-actin fibers. Based on
these results, a model of cytoplasm as a densely entangled filament network interpenetrated by a fluid phase crowded with globular macromolecules was developed which in good agreement with cell structure obtained from Medalia et al. (2002) in Figure 5.2C.

5.2 Materials and methods

5.2.1 Materials

The details of the synthesis and purification of the sixth generation poly-lysine dendrimer, (Gly)(Lys63)(NH2)64, molecular weight 8149 Da, are described in Chapter II. Hoechst 33352 (trihydrochloride trihydrate), wheat germ agglutinin- Alexa Fluor® 595 conjugate, Texas Red® transferrin and Citiflour® mounting medium were purchased from Molecular Probes, Netherlands. 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma, UK. Plasmid DNA, pDsRed2-N1 (4.689 Kb), was purchased from Clonetech, USA. The Label IT® Tracker™ Fluorescein kit was purchased from Mirus Bio Corporation, USA. OptiMEM reduced serum medium, gentamicin solution (10 mg/ml), penicillin/streptomycin (100 unit), MEM non-essential amino-acids solution (MEM), foetal bovine serum (FBS), phosphate buffer saline (PBS) without calcium and magnesium ions (D-PBS) (pH 7.5), Hank’s balanced salt solution (HBSS), Trypsin/EDTA were purchased from Gibco, UK. Human colon adenocarcinoma cell line (Caco-2), human embryonic kidney cell line (HEK-293), human Caucasian lung squamous carcinoma cell line (SKMES-1) and rat embryonic fibroblast cell line (REFS-2) were purchased from the European Collection of Cell Cultures (ECACC), UK.
Figure 5.2 Experimental evidence illustrates the existence and nature of macromolecular crowding in mammalian cells (A-B) and cryoelectron tomography of Dictyostelium discoideum cell cytoplasm (C). The translational diffusion of fluorescein labeled, size-fractionated Ficoll (FITC-Ficoll) probe was measured by fluorescence recovery after photobleaching in Swiss 3T3 fibroblast cells (A) and in vitro (B). $D_{\text{cyto}}/D_{\text{aq}}$ is the rate of diffusion in cells or test solutions relative to that in dilute aqueous solutions. In cells diffusion is already limited with the smallest size probe and it falls progressively with probe size. In vitro (B) tangle of F-actin filaments has little effect on diffusion of the smallest size probe but retards diffusion progressively with larger probes. In contrast, a high concentration of Ficoll polymer retards diffusion substantially but independently of probe size. The effects of F-actin and Ficoll are additive as the combination of these two (B) produces effects similar to that in cells (A). The interpretation is that the cytoplasm is a densely entangled filament network interpenetrated by a fluid phase crowded with globular macromolecules (Hou et al. 1990; Luby-Phelps et al. 1987). C showing the computational reconstruction of the cytoplasm of a Dictyostelium discoideum cell imaged using cryoelectron tomography. In this visualisation the actin filaments (red), membranes (blue), and cytoplasmatic complexes, mostly ribosomes (green) can be appreciated. The 2D projection corresponds to a volume of 815 x 870 x 97 nm (Medalia et al. 2002).
5.2.2 Methods

5.2.2.1 Fluorescent characterisation, optimization and cytotoxicity study

5.2.2.1.1 Confocal lambda analysis

The dendrimer possesses a broad emission spectrum (Figure 5.3) and may interfere in the emission spectrum of other fluorescent probes. To overcome this interference, the excitation and emission spectrum of the dendrimers were analysed by spectrofluorimetry as well as by confocal microscopy in order to select the optimum concentration of dendrimer which is free of such interference.

Figure 5.3 The emission spectra of various dendrimer concentrations, 3-600 μg/ml or 3.7x10^{-7}-7.4x10^{-3} mM, in water; at λ_{excitation} of 453 nm the peak of the emission was shifted to the lower wavelengths at concentrations >80 μg/ml (10 μM). The excitation and emission band width was 10 nm. (Al-Jamal et al. 2006)

Caco-2 cells were routinely cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 1% (w/v) non-essential amino acid solution (NEAA), 10% (v/v) foetal bovine serum (FBS) and gentamicin (50μg/ml) at 37°C under an atmosphere of 95% air and 5% CO_{2}. Cells were then resuspended in DMEM and seeded at a density of 1 x 10^{4} cells/cm^{2} on polylysine-coated glass cover slips in 12-well culture plates for 24 h. The cells were incubated in the complete media containing 0.1-0.5% dendrimers for 2 and 4 h and were excessive rinsed with serum free media, and ice cold D-PBS. Finally the cells were fixed with 5% paraformaldehyde and counter stained with DAPI before analysing by laser scanning confocal microscopy. For a control, the cells were incubated in complete media...
without the dendrimer and with/without staining. The specimen slides of the cells were scanned for emission spectra using excitation of 405 and 488 nm. The dendrimers and DAPI were detected by using excitation wavelength of 488 and 505 nm, respectively.

5.2.2.1.2 Cytotoxicity of the fluorescent labelling in cells (MTT assay)

Bisbenzimide Hoechst 33342 (Hoechst 33342) is a specific fluorescent stain for the AT-rich region of double-strand DNA and was employed for the uptake study in living cells. Although Hoechst 33342 is used worldwide in sorting living cells based on DNA content and in flow cytometry, its cytotoxicity has also been reported when it was used at concentration ≥ 5 μg/ml (Gregoire et al. 1984). To date, no specific information of cytotoxicity of Hoechst 33342 on Caco-2, SKMES-1 cell lines has been reported. The mistreatment of this substance may create cytotoxic effect on cells which can cause an alteration in their uptake capacity. This alerts us to ensure that Hoechst 33342 has not got any toxic effect on cells.

The cytotoxicity of Hoechst 33342 in Caco-2 and SKMES-1 cells was elucidated using an MTT assay (Mosmann 1983) - following the procedure explained in section 4.2.2.4 in Chapter IV with little modification. After seeding (1x10^5 cells/well) for 72h at 37°C, the cells were then incubated, for 4, 8, 16 and 24 h, with the desired concentrations, 2.5-10 μg/ml of the Hoechst 33342. Next, the medium was removed and fresh medium 150 μl was added to each well, followed by 10 μl of 5 mg/ml MTT solution. The cells were incubated for another 2.5 h at 37 °C in dark conditions. Lastly, the plates were inverted to remove the medium and gently tapped onto absorbent paper. DMSO, 200 μl, was added to each well before the plates were placed on a rotary shaker for 20 min to solubilise the crystals of formazan salt. An absorbance of the sample and the control in each well was determined at 550 nm and 630 nm using a plate reader. The results were expressed as the percentage viability (n=5; ±SD) calculated following equation 4.2 (see Chapter IV).
5.2.2.2 DNA labelling, formulation and characterisation of dendriplexes

5.2.2.2.1 Fluorescent labelling of DNA

Plasmid DNA (pDNA), pDsRed2-N1 (4.689 Kb), obtained from section 4.2.2.1.2 (in Chapter IV) was employed in this study. The pDNA was fluorescently labeled using Label IT® Tracker™ Fluorescein kit (Mirus Bio Corporation, USA). The kit is based on Mirus’ propriety nucleic labelling technology which initiates the covalent bond between the label and guanine residues at the N7 in a non-destructive manner of DNA. The labelling procedures were carried out according to the manufacturer’s instruction (Label IT® Tracker™ Protocol (Mirus Bio Corporation 2005)) which are summarized in Figure 5.4. The kit containing three reagents: Label IT® Tracker™ reagent (R1), Tracker reconstitution solution (R2) and 10x Labelling buffer A (R3). First, 50 μl of R2 solution was added to Label IT® Tracker™ pellet to make up the R1 solution before the labelling process was began.

Sterile water (37.5 μl) and R3 buffer (5 μl) were added into 5 μl of 1 mg/ml pDNA. The solution was mixed briefly and the R1 solution, 2.5 μl (0.5:1 (v:w) ratio of Label IT® Tracker™ reagent to DNA) was finally added to the mixture and mixed briefly to achieve final DNA concentration at 0.1 mg/ml. The reaction was performed at 37°C for 1 h (1). The labeled DNA (LDNA) was next purified by ethanol precipitation; 0.1 volume of 5 M NaCl and 2 volumes of ice cold 100% endotoxin free ethanol were added into the reaction tube (2). The solution was mixed well and kept at -20°C for 1 h. The reaction tube was then centrifuged at full speed in refrigerated microcentrifuge for 10 min (3) to make LDNA pellet (4). A room temperature 70% endotoxin free ethanol, 500 μl, was added to wash the pellet and the mixture was centrifuged at the same condition as mention above for 10 min before the ethanol was carefully eradicated. The LDNA pellet was resuspended in 10 μl of 1x Labelling buffer A or sterile water and store at -20°C until used (5). The LDNA was characterised using agarose gel retardation assay in 0.8% agarose gel (see 5.2.2.2.3, Chapter IV).
Adding sterile water
Adding R3 solution
Adding R1 solution

Incubating at 37°C for 1 h

DNA solution
(1 mg/ml), 5 µl

DNA labelling reaction

Purification of labelled DNA

Adding 0.1 volume of 5 M NaCl
Adding 2 volumes of ice cold 100% endotoxin free ethanol
Placing at -20°C for 1 h

Rinse DNA pellet with room temperature 70% endotoxin free ethanol

LDNA Pellet

Centrifuges at 15,000g, 4°C for 10 min
Removes the supernatant

DNA Precipitation

Centrifuges at 15,000g, 4°C for 10 min
Removes the supernatant
Resuspends in 1x Labelling buffer A or sterile water

Fluorescent-labelled DNA stock solution (LDNA)

Figure 5.4 A summary of the fluorescent labelling procedures for plasmid DNA with Label IT® Tracker™ Fluorescein kit: the labelling reaction was carried out at 37°C for 1 h (1). Next, the labeled DNA (LDNA) was purified by ethanol precipitation (2) before the LDNA was separated by centrifugation (3). The pellet of LDNA was rinsed with 70% ethanol (4) and resuspended in 1x labelling buffer A or sterile water (5). The solution of LDNA was kept at -20°C until used.
5.2.2.2 Formulation and characterisation of fluorescent-labelled dendriplexes

The fluorescently labelled DNA (LDNA) obtained from section 5.2.2.2.1 was used to formulate dendriplexes. The dendriplexes (DM1-LDNA complexes) were prepared following the method described in section 4.2.2.2 in Chapter IV. The complexes were incubated at 25°C for 30 min before being used.

5.2.2.2.3 Physicochemical characterisation

The LDNA binding capacity of dendrimer (DM1) was analysed using 0.8% agarose gel electrophoresis in Tris borate EDTA buffer (TBE buffer) as explained in 4.2.2.3.1 (Chapter IV) except not adding ethidium bromide into the gel during casting the gel but the gel was stained with the ethidium bromide after electrophoresis. The hydrodynamic diameter of the dendriplexes (DM1-LDNA complexes) was elucidated using photon correlation spectroscopy (Autosizer 4700, Malvern, UK) according to the procedure described in 4.2.2.3.2 in Chapter IV. The zeta potential of the dendriplexes were analysed by Zetasizer 3500 (Malvern, UK) as mentioned in section 4.2.2.3.3 in Chapter IV. The dendriplexes were also characterised by transmission electron spectroscopy (TEM, Philips CM 120, Einhoven, Netherlands) following the procedure also explained in 4.2.2.3.4 (Chapter IV).

5.2.2.3 The uptake study of fluorescent poly-lysine dendrimers and dendriplexes

In this section the experiments were attempted to investigate the uptake of the dendrimer by individual adhering cells. To ensure that the monolayer were not formed, the cells used here were seeded and grown at the lower concentrations and shorter period of time than that in MTT experiment, and they were checked under the microscopy before used.

5.2.2.3.1 Uptake of the dendrimer and dendriplexes using fixed cells

Cells were routinely cultured in standard procedures as mentioned in 5.2.2.1.1 before they were seeded at the same density (1x10^4 cells/well) on polylysine-coated glass cover slips in 6-well culture plates. After seeding for 24 h, the cells were incubated with the complete medium containing desired concentrations of the dendrimer or
dendriplexes for different periods of time (t). The cells were incubated with the desired concentrations of the dendrimer which can be used without cytotoxicity, that is, less than 5 mg/ml with no longer than 4 h of incubation (see Chapter IV section 4.3.3). At t = 0 min, 15 min, 30 min, 1 h, 2 h, and 4 h the cells were rinsed with serum free media, and ice cold D-PBS (x2). The cells were fixed with 4% paraformaldehyde for 5 min and the reaction quenched with 5 mM ammonium chloride for 10 min. The cells were then extensively rinsed with iced cold PBS and counter stained with 300 nM of DAPI for exactly 5 min. The cells were, again, rinsed with iced cold PBS (x2) before mounting on slides with antifading media, Citifluor™. For controls, cells were incubated in the same way without dendrimer and fixed as described above. The specimens were finally observed under confocal laser scanning microscopy (CLSM) using multi-track mode with LSM510 software.

5.2.2.3.2 Uptake of the dendrimer and dendriplexes within living cells

To study uptake in living Caco-2 and SKMES-1 cells, a similar procedure described in 5.2.2.3.1 was carried out except that the cell nuclei were stained with Hoechst 33342 by incubating with DMEM containing 5μg/ml of Hoechst 33343 for Caco-2 cells or 2.5μg/ml of Hoechst 33342 for SKMES-1 and REFS-2 cells for exactly 30 min prior to the experiments. The cover slips containing the cells were then transferred into a perfusion chamber and the cover slip was fixed with silicone grease to ensure that there is no movement during imaging. Z-stack images of the cells were taken every 5 min using CLSM before and after the dendrimer were added into the media. After the experiment the cells were re-incubated and viability of the cells was assessed. The data were analysed as the following

5.2.2.3.2.1 Image and data analysis

The model of data analysis here was assumed to study the uptake of the dendrimer/dendriplexes by the individual adhering cells without a mono layer condition. Therefore, most of the cells selected in this experiment were one individual cell or a group of a small amount of cells.
Dynamic uptake of the dendrimer in all types of cells was evaluated using LSC Lite software and custom procedures written in IgorPro software (Wavemetric Inc). The z-stack image at time t=5 min before the dendrimer or the dendriplexes was combined and used as control image. The fluorescent intensity detected of green channel (Ch2) in this stack represents the background fluorescent intensity. The regions of interest (ROIs) were drawn and saved as the reference file. The fluorescent intensity of the dendrimer/dendriplexes (Ch2) inside each z-stack images were averaged and standardized by background subtraction.

The diffusion coefficients (D) and the diffusion coefficients related to those in water (D/Do) of the dendrimers and dendriplexes were calculated. The D value was obtained from equation 5.6 (Martin 1993).

\[ t_L = \frac{h^2}{6D} \]  

where \( t_L \) is the lag time and assuming the cytoplasm is homogenous. Considering the transport of the dendrimer and dendriplexes from the plasma membrane to the nucleus, there is “lag time” (\( t_L \)) for the dendrimers and dendriplexes to develop a uniform concentration gradient within the cytoplasm which allows calculation of a diffusion coefficient, where the thickness (h) of the diffusion layer is known. This thickness is the mean distance between the plasma membrane and the nuclear membrane, termed the “cytoplasmic radius”. An average value must be used as cell dimension vary in xy-plane. There are two reasons for not taking the z-plane diffusion in to an account. First, the cells used in this experiment are a single or small group of cells which can be depicted in Figure 5.21A. These cells have only small distance between the cell membrane and nuclear membrane compared to that in xy-plane under microscopy, and thus the cytoplasmic radius in z-plane can be neglected. Unlike the monolayer system (the cells begin to transform into the cubical shapes) in which the nuclei of the cells mostly locates close to cell base reflecting the significant distance of the cytoplasmic radius in z-plane. In case of the monolayer system this assumption might not be suitable. Second, even at the highest magnification of the objective lens of the confocal microscopy the detail of the z-plane can not be processed due to the

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capacity of the machine. To meet the validity of these analysis assumptions the conditions of cells used in the study are crucial.

The cytoplasmic radius was calculated as mentioned in section 5.2.2.3.2.2. The value of \( t_L \) was obtained by measuring the fluorescence intensity of the dendrimer over time from uniformly sized regions of interest placed equidistantly across the cell using custom procedures written in IgorPro software. The diffusion coefficient (D) of the dendrimer in the cell cytoplasm was then calculated, using the experimental \( t_L \) value. The set fluorescent intensity of ROIs in each time point (F) was then plot against the time (min).

5.2.2.3.2.2 Assessment of the “cell radius”

In order to calculate the diffusion coefficient of the dendrimer or dendriplexes in cell cytoplasm the distance between the plasma membrane and the nuclear membrane – called here the “cell radius (h)” (Figure 5.5) - of living cells were measured by staining the cell nuclei and the plasma membrane with Hoechst 33342 (5 or 2.5 \( \mu \)g/ml as mentioned in 5.2.2.3.2) and germ wheat agglutinin (WGA), 5\( \mu \)g/ml, respectively. Cells growing in the same condition described in 5.2.2.3.2 were incubated in Hanks’ Balanced Salt Solution (HBSS) containing desired concentration of Hoechst and WGA for 30 min and excessively rinsed with PBS. The cells were then transferred into the perfusion chamber and the z-stack images of the cell were made using CLSM. The distance between the plasma membrane and nucleus of 50 cells in each type of cells were measured from the average of \( h_1 \) and \( h_2 \) (Figure 5.5) using Leica confocal software (LSC Lite) and the average distances (n=50) were calculated.
The distance between cell membrane and nuclear membrane was also confirmed using TEM. Cells growing on cover slips were fixed with 2.5% glutaraldehyde in PBS for at least 4 h at 4°C and rinsed with PBS (x3). The specimen was kept in PBS at 4°C until ready to process. The sample was post-fixed in osmium tetroxide solution (See in Appendix III) for 15-30 min in the fume cupboard. The cover slips were dehydrated in 10% (x1) ethanol in PBS for 10 min at room temperature. The cover slips were further dehydrated with 70% (x1) and 100% (x3) ethanol in PBS at room temperature for 15 min for each step (in a total period of 1 h). The processing code of each cover slip was made on the glass slide before it was placed in the slide tray. A small drop of TAAB premix medium resin was added onto the slide to hold the cover slip in place. The cover slip was lifted from the well and placed on the slide which was immediately covered with a few drops of the resin. Care must be taken as the cover slips are not allowed to dry out. The slide tray was leaved in fume cupboard for 2 h.

The TAAB beam capsule was filled into the rack, one for each cover slip. Each capsule was then filled with the resin to the brim. The beam capsule was neatly inverted onto the cover slip in the tray to embed the cells into the resin. The tray of slide and beam capsule was placed into the embedding oven and leaved the resin to polymerise for 24 h. The polymerized resin preparation was removed from the oven.
and leaved to cool down. The hot plate was warmed up for 20 min. The resin preparation was placed on the hot plate for 30 sec and the beam capsule was snapped off from the cover slip before it was placed into its prepare embedding box. The beam capsule embedding cell monoculture was cut on Leica Ultra-cut machine (Leica UK Limited) into semi-thin thickness, 0.75-2 μm. The obtained semi-thin sections are stained with toluene blue and viewed to select the target area under TEM (Hitachi H7600, Hitachi High-Technology, UK). The section was placed on a TEM grid and stained with uranyl acetate and lead citrate before viewing.

5.2.2.3.3 The influence of fluid flow on the uptake of the dendrimer and dendriplexes

The uptake study was carried out as described above (section 5.2.2.3.2) except that the medium was circulated and introduced into the perfusion chamber at a flow rate of 2 ml/min. The experimental set up is demonstrated in Figure 5.6. After the cells were transferred to the perfusion chamber the desired concentration was injected into the inlet and the cells were exposed to the dendrimer/dendriplexes for 5 min before the medium was circulated using pump. Dynamic uptake of the dendrimer by cells was recorded by CLSM as previously explained in section 5.2.2.3.2 and the data were analysed according to the method mentioned in section 5.2.2.3.2.1.

5.2.2.3.4 Assessment of the uptake pathways of the dendrimer and dendriplexes

Texas Red® transferrin (Molecular Probe) is a fluorescently labeled transferrin that was used in this study. Since the transferrin (MW ~80 kDa) usually binds to Fe³⁺ ions for delivery to vertebrate cells through receptor-mediated endocytosis, therefore, its fluorescent conjugated form can be used as a probe for the investigation of endocytosis.
Figure 5.6 Schematic diagrams showing the experimental setup to investigate an influence of fluid flow on uptake of dendrimer and dendriplexes. The perfusion chamber was inserted in the heated stage of confocal microscopy and the temperature was maintained at 37°C (A). Dendrimer was first injected into the chamber inlet (orange) and the cells were allowed to expose to the dendrimer for 5 min (B). Then the pump was switch on to regulate the circulation of the medium through the chamber. The reservoir of DMEM containing dendrimer or dendriplexes at the similar concentration as previously injected into the inlet was circulating through the chamber with flow rate of 2 ml/min as explained above. The reservoir was aspirated with 5% CO₂ at all the time. The data were recorded and processed by the computer connected to the confocal microscope as described in 5.2.2.3.2.1.

The cells were first incubated with the DMEM containing 1.0% Bovine serum albumin (BSA) for 45 min, at 37°C under an atmosphere of 95% air and 5% CO₂, to remove the endogenous transferrin from the receptors. Next, the uptake study was performed as explained in 5.2.2.3.2. At the time (t) of t= 30 min before termination BSA and transferrin in DMEM was added into each well to achieve final concentration of 0.5% of BSA and 50μg/ml of transferrin, respectively. Cells were fixed with 4% paraformaldehyde as described in 5.2.2.3.1 before the cover slip was viewed under CLSM. The maximum excitation and emission wavelengths of Texas Red® transferrin were 595 and 615, respectively.
5.3 Results and discussion

5.3.1 Characterisation of intrinsic fluorescent dendrimer

5.3.1.1 Confocal lambda analysis

As information about the excitation and the emission spectra of these poly-lysine dendrimers was not available, we investigated the emission spectrum of the dendrimers using excitation wavelengths at 405, 453, 458 and 488 nm (Figure 5.7). The excitation wavelengths at 405, 458 and 488 nm are the only excitation wavelengths that can be used in our confocal microscopy. The aim of this study was to examine the emission spectrum of dendrimers at different excitation wavelengths and to be able to select the proper fluorescent tag to allocate the cell position.

Generally, the dendrimers have very broad emission spectra at all excitation wavelengths. The fluorescence of dendrimers is concentration dependent which corresponded with the results of the previous study by Al-Jamal et al (2006). In their experiments, the maximum emission wavelength of the identical dendrimer in distilled water was 514 with the excitation wavelength of 453 nm. It was found that, the maxima emission spectra of dendrimer were shifted from 470 nm to 490, 496 and 520 when the excitation wavelengths were 405, 453, 458 and 488, respectively (Figure 5.7A-D). The use of PBS as a medium in our experiment might change the pH of the suspension of dendrimers which could make the dissimilarity of the maxima emission spectrum.

The DAPI fluorescent tag, 4'6-diamidino-2-phenylindole dihydrochloride, was selected and was used to locate the cell nucleus when the uptake study of dendrimers were performed. The maxima emission and excitation wavelength of DAPI-DNA complex is 454 and 364 nm respectively and the dendrimers was detected at the excitation wavelength of 488 nm. The lambda analysis of the DAPI-staining and the fixing cells were also done under confocal microscopy and the results are illustrated in Figure 5.8 - 5.9. Figure 5.8 shows the fluorescent micrograph and the emission spectrums of the fixed and non-staining cells (Figure 5.8A) recorded at the excitation wavelength of 405 (Figure 5.8B) and 488(Figure 5.8C) nm.
Figure 5.7  The emission spectra of various concentrations of dendrimer when the dendrimer was excited at wavelengths of 405(A), 453(B), 458(C) and 488(D) nm, respectively.

As the cells were fixed only with 4% paraformaldehyde, the emission spectra thus represented in part the fluorescent background of the fixing. At an excitation wavelength of 405, there is a small background noise with maxima emission at about 485 nm while that was not found in the emission spectrum recorded at the excitation wavelength of 488 nm. Staining cell nucleus with DAPI (Figure 5.8P), the maximum emission wavelength of DAPI was found to be at about 456 nm (Figure 5.8Q) and DAPI per se was not found to emit at the excitation wavelength of 488 nm (Figure 5.8R). The maximum mission wavelength of DAPI from the experiment was very close to the maxima emission wavelength received from the company, 454 nm.

In Figure 5.9 the florescent micrographs of the cells incubated with dendrimers for 2 h before fixing and staining with DAPI were recorded at the excitation wavelength of 405 (A) and 488 (B) nm. It was found that a small amount of dendrimer was taken up. From the emission spectra, Figure 5.9C there is no shifting of the maximum emission
wavelength of DAPI when dendrimers were added into the system. Although dendrimers did not show their maximum emission spectrum due to its low concentration, the signal of the dendrimers was higher than that of the background (Figure 5.9D). Nonetheless, it was noticed that the intensity of the background was double when the dendrimers were added into the system. This might be because of the fluorescent signal of some of the dendrimers in cell cytoplasm. These results imply that DAPI can be used to locate the cell when the uptake study of dendrimers was performed. Figure 5.10A shows fixed and stained specimen of the dendrimer after incubating with the medium (DMEM) containing 0.05% (w/v) of dendrimer (DM1) for 4 h. The merged channel was magnified in B. The results suggested that dendrimers were taken up into the cells and some of them could travel to the nucleus (A-B). This is evidenced by the colocalisation of the DAPI (blue, Ch1) and dendrimers (green, Ch2) inside stack section within the cell nucleus in merge channel (Ch3).

The plot in Figure 5.10C illustrates the cumulative fluorescent intensity (CF) of DAPI nucleus staining throughout whole stack of images. The cumulative fluorescent intensity of the dendrimers inside the nucleus presenting as all ranges of the stack images can be seen in Figure 5.10D when the specimen was excited at $\lambda_{488}$. These results imply that the optimal concentration range of the dendrimer which can be used as a nano-fluorescent probe in the uptake study is 0.05-0.1 % (w/v), since at this concentration range, there is no interference in emission spectra between the dendrimer other fluorescent probes (here DAPI). On the contrary, there have also a chance of the co-emission of the dendrimer in ch1 (blue) as the dendrimer having broad excitation and emission spectrum, although the concentration of the dendrimer used was considering low. This can be explained by the same pattern of the fluorescent signal were detected in both channels. As the study was performed in fixed cells the comparison between before and after adding the dendrimer within the same specimen can not be carried out; the further study on the dynamic uptake in live cells are recommended to assess this unclear.
Figure 5.8  The fluorescent micrographs and the emission spectra of the fixed non-staining cells (A-C) and fixed DAPI-staining cells (P-R) recorded at the excitation wavelength of 405, $\lambda_{405}$, (B and Q) and at 488 nm (C and R). The peak of the fixing background is detected at $\lambda_{485}$ (B) while no peak is detected at excitation $\lambda = 488$ (C). The peak of DAPI is clearly shown at the $\lambda_{456}$ (Q) and no peak is found at excitation $\lambda = 488$ (R).
Figure 5.9 The fluorescent micrograph of the fixed and DAPI-staining cells incubated with dendrimers, 0.05% w/v, for 2 h recorded at the excitation wavelength of 405(A) and 488(B) nm and their emission spectra (C and D), respectively. The peak of DAPI is shown at $\lambda_{456}$ with no interference from the dendrimers.
Figure 5.10  Fluorescent micrograph (A-B) and the emission spectra of the fixing and DAPI-staining cells, after incubation with dendrimers for 4 h, recorded at the excitation wavelength of 405 (C) and 488 (D) nm. There is only one peak of the fluorescent intensity detected in each excitation wavelength. This reinforces the view that there is no cross-emission between DAPI and dendrimers.
5.3.2 Cytotoxicity study of fluorescent staining Hoechst 33342

Results of cytotoxicity of the fluorescent staining agent, Hoechst 33342, in Caco-2 and SKMES cells at various incubation periods is demonstrated in Figure 5. The toxicity effect of Hoechst 33342 depends on incubation period, Hoechst concentration and cell line. In general, the increase in concentration and incubation period resulted in an increase in the toxic effect of Hoechst 33342. A slight toxicity was found in Caco-2 cells (A) whereas the greatest effect was found in SKMES-1 (B). The concentration range of Hoechst 33342 that can be used in the uptake experiment is 1-10 µg/ml and 1-3 µg/ml in Caco-2 and SKMES-1 cells, respectively, for the maximum incubation period of 8h. To make sure that the selected concentration Hoechst 33342 exhibits an efficient fluorescent signal, various concentrations within those ranges was optimized under confocal microscopy. The results indicated that the optimal concentrations of Hoechst 33342 in SKMES-1 and Caco-2 cells are 2.5 and 5 µg/ml.

5.3.3 Formulation and characterisation of dendrimer-LDNA complexes

5.3.3.1 DNA labelling and dendriplex formation

The binding capacity of the dendrimer (DM1) to the fluorescent - labeled DNA (LDNA) and LDNA labelling with different fluorescent probes, Yoyo-1 and Mirus Green, were characterised using 0.8 agarose gel retardation assay is demonstrating in Figure 5.12. The fluorescein signal from the labeled DNA in the unstained gel is shown in panel A. Although Mirus green formed covalent bonds to the guanine residues of the DNA (5), it caused no shift in the DNA band (lane 5, A-B). In contrast, the binding of Yoyo-1 showed a slight fluorescent signal (3-4) and caused the shift of the band in panel B. The reason for such differences is due to the different binding mechanism between the fluorescent probes and the DNA. Yoyo-1 intercalated with minor groove of the DNA which may cause a change in DNA conformation and total charge presenting as a shift of the DNA band in B (3-4). For dendriplex formation, the dendrimer exhibits complete condensation of the labelled DNA at a molar charge ratio of 10:1 (+/-) as can be seen in panel B (6-8). As the parent DNA is the same as the DNA used in the transfection study in Chapter IV (the 10:1 ratio)
which displayed high transfection efficacy, this molar charge ratio was therefore selected here for complex formation and characterisation.

**Figure 5.11** Estimates of the cytotoxicity of Hoechst 33342, a fluorescent staining agent. The toxicity was evaluated using a MTT assay at various incubation periods: 4, 8, 16 and 24h. The results indicate the safe concentration ranges of Hoechst 33342 which should be used in both Caco-2(A) and SKMES-1(B) cells which are at 1-10 and 1-3 μg/ml, respectively, for the maximum incubation of 8 h. To ensure that there is no toxic effect of Hoechst 33342 and fluorescent signal is efficient, the uptake experiments of the dendrimer and dendripplexes were performed using 5 and 2.5 μg/ml of Hoechst 33342 in Caco-2 and SKMES-1, respectively, with 45 min of incubation period.
12.210

Figure 5.12 Agarose gel retardation assays of fluorescent-labelled DNA and the dendriplexes before (A) and after the same gel is stained with 0.5µg/ml ethidium bromide in TBE buffer (B); the DNA was labeled with Yoyo-1 (3-4) and Mirus Green (5) fluorescent probes ($\lambda_{em}$ = 488, $\lambda_{em}$ = 514 nm). The fluorescein signal from the labeled DNA in the unstained gel is illustrated in panel A. The covalent labelled DNA by Mirus green (5) shows no shift of the DNA band (B). The Yoyo-1-labelled DNA shows a slight fluorescent signal (3-4) with the shift of the band in panel B. Yoyo-1 intercalates with minor groove of the DNA causing the shift of the DNA band in B (3-4) due to the alteration in the charge of the DNA. The complete condensation of the labelled DNA is found at charge ratio of 10:1 (+/-) as can be seen in panel B (6-8).

The data suggest that the Mirus Green fluorescent probe should be selected as a probe for DNA in the uptake study of the dendriplexes because there is no shift and no condensation of the DNA in comparison to that of Yoyo-1-labelled DNA.

5.3.3.2 Characterisation of the dendriplexes

The hydrodynamic diameter of the dendriplexes was found to be 107.33 (SD ± 1.53) nm with polydispersity index of 0.259 ± 0.009 (Figure 5.13A). This is in good agreement with the result obtained from TEM in (Figure 5.13B) and compares with that of the non-labelled DNA that is complexed at a 10:1 (+/-) ratio, namely 84.60 (SD ± 1.51) nm. The diameter of DM1-EDNA complexes is increased by 23 nm (Figure 5.13A). The zeta potential of the complexes between the fluorescent labelled DNA and the dendrimer increases to 39.80 (± 1.65) mV from the values for the non-
labelled DNA complexes, 23.47 mV. Such alteration may be explained by the changes in the DNA structure after it was labelled. The covalent linkage between the fluorescent probe and the DNA may increase in molecular volume and enhance steric hindrance of the labeled DNA molecules. This could significantly affect the binding between the DNA and the dendrimer.

Similarly, an increase in the zeta potential of the DM1-LDNA complexes may be due to the covalent linkage of the fluorescent probe. This link, perhaps, can cause DNA condensation render the enclosure of the negative charges of the DNA molecule and this result in a reduction in the total charge of the labeled DNA. When this labeled DNA is assembling with the dendrimer, the labeled DNA could, therefore, neutralise smaller amounts of the positive charge of the dendrimer. The decrease in the negative charge of the labeled DNA and the increase in the steric hindrance regarding the covalent link of the fluorescent probe may render the binding between the labeled DNA and the dendrimer looser as evidenced by the increased diameter of the dendriplexes in Figure 5.13 A.
Figure 5.13 The physicochemical properties of the dendriplexes of non-labelled and Mirus green labeled DNA: the particle diameter and zeta potential of the dendriplexes (A) increase by 20 nm and 15 mV after the DNA was labeled with the fluorescent probe and subsequently formed complex to the dendrimer (DM1). These increases might be due to the changes in the structure after the DNA was labeled. The covalent link between the probe and the DNA may result in an increase in molecular volume and steric hindrance together with the decrease in the zeta potential of the DNA (see text). Such increase is also confirmed by the result obtained from the transmission electron microscopy as shown in B.
5.3.4 Uptake of dendrimer and dendriplexes

5.3.4.1 Uptake of dendrimer and dendriplexes in fixed cells

The uptake of the dendrimer in Caco-2 cells is time dependent as illustrated in Figure 5.14 and 5.15. At 15 min, the dendrimer (seen by the green fluorescence) concentrates at the periphery of cell membrane (Figure 5.14B). Within 30 min, the dendrimer is found to accumulate in the cytoplasm (Figure 5.14C). The relatively rapid uptake into the Caco-2 cells and specifically the nucleus can be attributed first to due to the electrostatic attraction between the amino surface groups of the dendrimer and the negative charge of membrane proteins, which allows attachment to the cell membrane prior to endocytosis. The journey of the dendrimers to the nucleus then begins. Within an hour, the dendrimer is found in all parts of the cell (Figure 5.14D-E). Distribution of the dendrimer toward cell nucleus are observed in dividing (D) and non-dividing cells (E). The dendrimer was found all over the cells and more concentrated in the cytoplasm (Figure 5.14F-G). The cells after exposure to the dendrimer for 4h are demonstrated in Figure 5.14H, the higher amount of dendrimer patches are found all over the cells.

To confirm nuclear uptake of the dendrimer, the cell nuclei were stained with DAPI (blue, Ch1) before fixing. At zero time, following rinsing and fixation, a slightly background fluorescence was observed in the cells (Figure 5.15A). Thereafter it was seen that the longer the incubation period the higher the signal derived from the dendrimers (green, Ch2), Figure 5.15B-F. Dendrimers (green) were observed near the nuclear membrane after 15 min (B). The dendrimer was obviously found in the nucleus identified by DAPI staining (blue). These results suggest that dendrimers were taken up by the cells and some could reach the nucleus within 30 min of incubation. The rapid uptake of the dendrimer into the Caco-2 cells and the nuclear compartment might be initially due to the electrostatic attraction as mentioned above. As far as we aware there are as yet no quantitative reports of this phenomena. Once in the cytoplasm, the small size of dendrimer particles (about 6.5 nm in diameter) might allow the dendrimers both to diffuse rapidly and to pass easily through the nuclear pores, which have a diameter of about 9-10 nm. Further studies of the dynamics of
uptake of the dendrimers and dendrimers-DNA complex in living cells, is discussed in the next section.

Figure 5.14 A time dependent uptake study of the dendrimer (green fluorescence) in Caco-2 cells: the cells were incubated with complete media containing DMEM supplemented with 1% (w/v) MEM, 10% (v/v) FBS, gentamicin (50 mg/ml) and 0.1% (w/v) of dendrimers for 15 min (B), 30 min (C), 1 h (D-E), 2 h (F-G) and 4 h (H). The cells were rinsed and fixed with 4% paraformaldehyde before observing under confocal microscopy. In control (A), the cells were exposed to the completed medium without the dendrimer. The dendrimer is found to attach to the cell membrane periphery (red arrows) as a result of electrostatic attraction between the negative charge of the cell membrane and positive charge of the dendrimers (B-C). At 1 h the dendrimer distributed towards the cell nuclei as it can be seen in all regions of the cells (D-E). At the longer incubation period (F-H), the dendrimer appears to be concentrated on the surface of the cells. This technique cannot prove internalization.
Figure 5.15 The “stack” images of dendrimers in Caco-2 cells after the cells are exposed to dendrimer at different periods of time: 0 min (A), 15 min (B), 30 min (C), 1 h (D), 2 h (E), and 4 h (F). The uptake of the dendrimer is a time dependent process. The dendrimer was readily found at the perinuclear membrane after 15 min and in the nucleus after 30 min incubation. Bars are 8 μm in A and C-F, and 12.7 μm in B.
However, different results were found in another cell line, SKMES-1 (Figure 5.16). The dendrimer appeared randomly to adhere or adsorb on the cell membrane as can be seen in Figure 5.16J-L. The reasons for this variation remain unclear the study of the interaction between the dendrimer and cell membrane from different types of cell lines may shed light on this mystery. At longer incubation periods a more typical trend was found: the dendrimer was found to cover the cell as seen in images M-N.

![Image](https://example.com/image.png)

**Figure 5.16** A time dependent uptake study of the dendrimer (green fluorescence) in SKMES-1 cells: the cells were incubated with complete media containing DMEM supplemented with 1% (w/v) MEM, 10% (v/v) FBS, gentamicin (50 mg/ml) and 0.1% (w/v) of dendrimers for 15 min (J), 30 min (K-L), 1 h (M) and 2 h (N). The cells were rinsed and fixed with 4% paraformaldehyde before observing under confocal microscopy. In control (I), the cells were exposed to the completed medium without the dendrimer. The dendrimer is found to randomly attach to the cell membrane (red arrows) as a result of electrostatic attraction between the negative charge of the cell membrane and positive charge of the dendrimers (J-L). At 1 h the dendrimer distributed to all regions of the cells (M). At the longer incubation period (N), the dendrimer is found more concentrate cover the cells.

**5.3.4.2 Uptake of dendrimer and dendriples in living cells**

As low concentration of the dendrimer used, the co-localisation of the dendrimer may not be very obvious, but the increase in the amount of the fluorescent signal of the dendrimer was found inside the cell nucleus after background subtraction. Interestingly, the some pattern was noticed in the nucleus after the dendrimer was
added into the system. This might be due to the dendrimer possesses a broad excitation and emission spectra and thus the emission of the dendrimer and it is, thus, possible to emit at the same excitation wavelength to the Hoechst 33342. This can be seen from the alteration of the blue fluorescent signal after the dendrimer was added (Figures 5.17A-C). As the Hoechst 33342 was only used for allocating the nuclear compartment and the alteration of the fluorescent signal in channel 1 has no effect on the fluorescent signal in channel 2.

The dynamic uptake of the dendrimer into the nucleus was examined by overlaying images of the dendrimers (in green channel (Ch2)) over images of the nucleus (in blue channel (Ch1) as can be seen in Figures 5.17-5.18. The fluorescent signal in the green channel, representing the dendrimers in each ROI, was normalized by the background fluorescent value (white boxes no. 14 in the insert pictures of Figure 5.17G and 5.18G). The relative fluorescent signal of the dendrimer was then plotted (F) against time (t, min) as in Figures 5.17G and 5.18G. The results (n=5) demonstrated that the dendrimer reached the nucleus of Caco-2 cells within 35-45 min (t_L) of incubation (Figure 5.17G), compared to 25-30 min in SKMES-1 cells (Figure 5.18G). This might be due to the differences of the intracellular architecture within each cell line and also to the size of the cells. The lag time (t_L) required for the dendrimer to develop a uniform concentration gradient within the nucleus was used to calculate a diffusion coefficient.

The cytoplasmic radius was found to be 12.09 (±6.02 (SD)) and 8.01 (± 3.21 (SD)) μm for Caco-2 cells and SKMES cells, respectively. The diffusion coefficients (D_cyto) of the dendrimer in both cell lines were calculated, using the experimental t_L value. D_cyto values of the dendrimer in the cytoplasm of Caco-2 cell and SKMES-1 were found to be 9.82 (± 0.98)x 10^{-11} and 5.99 (± 0.16)x 10^{-11} cm^2 s^{-1}, respectively. Cellular components such as secretory granules have diffusion coefficients of 1.9 x 10^{-11} cm^2 s^{-1}, even lower than that of the dendrimers (Kaether & Gerdes 1999) as might be expected from their larger dimensions. The diffusion coefficients relative to those in water (D_cyto/D_water) are, respectively, 1.24 (±0.12)x10^4 and 0.76 (±0.05)x10^4 (D_water =79.54x10^{-8} cm^2 s^{-1}). These values implied that macromolecular crowding and
obstacles in the cytoplasm provide a formidable barrier to dendrimer transport. The cytoplasm hinders the mobility of the dendrimer by 1000 fold compared to that in water.

This may be explained in loose terms by exclusion and obstruction effects (explained in Chapter III), the former due to the loss of free water by hydration of macromolecules and the latter due to the tortuous pathway that the dendrimer must travel in the cytoplasm avoiding organelles and other structural features, such as actin fibres. The diffusion of globular proteins (Arrio-Dupont et al. 2000) in muscle cells approximates to zero when the hydrodynamic radius of the protein approaches 7 nm (Tseng et al. 2004). This implies that the cytosol may behave like a gel which can restrict movement of the particles by a sieving effect. This is in accordance with the cell model proposed by Luby-Phelps et al (1993) which describes the cytoplasm as a densely entangled filament network of actin bundles (insoluble phase) interpenetrated by a fluid phase crowded with globular macromolecules.

Furthermore, we have evidence of an interaction due to the electrostatic attraction between the dendrimer and actin cytoskeleton in vitro, and have determined the diffusion coefficient of the dendrimer in the presence of actin gel using Fluorescent Recovery after Photobleaching (FRAP) technique (Chapter III). The ratio $D_{actin}/D_{water}$ of the dendrimer was found to be 0.26 which means that mobility of the dendrimer was reduced up to 75% in an actin gel in vitro. The concentration of actin gel was 1 mg/ml of actin whereas that inside the cells was 10 mg/ml. The viscosity inside the cells is, therefore, expected to be much higher than such actin gel. The viscosity of the cytosol is said to be 2.6 to 10 fold higher than water; the variation of the viscosity found here is, perhaps due to the differences in cell types and different techniques (Luby-Phelps et al. 1987; Kao et al. 1993; Luby-Phelps et al. 1986; Partikian et al. 1998; Swaminathan et al. 1996; Swaminathan et al. 1997). These viscosity values contributed to the retarded diffusion, although the medium in which particles diffuse approximates to that of pure water (Fushimi & Verkman 1991; Kao et al. 1993). Therefore, the auxiliary reduction of the translation diffusion of the dendrimers in cytoplasm can be attributed to microscopic barriers. Further studies on this are needed.
Figure 5.17 The overlay of the compressed z-stack images of the dendrimer uptake process in living Caco-2 cells. The cells were incubated with medium containing 0.1% dendrimer (represent in green) and images were collected every 5 min before (A) and after adding the dendrimer (B-F). Images presented here are at time t= 5(B), 15(C), 30(D), 40(E) and 90(F) min of incubation period. Data were analysed over the time and plotted in G and the insert pictures show the positions of regions of interest (ROIs, white boxes) for data analysis. The mean fluorescent signal in each ROI was measured from projected Z-stack images taken at different time points and generalized by background subtraction. The dendrimer was found in the cytoplasmic compartment in the period of 5-35 min after exposed to the dendrimer (ROI 9 and 10). The concentration of the dendrimer declined after 30 min whereas the relative fluorescent intensity of the dendrimer inside the nucleus was detected (ROI 5-8). The cell nuclei were stained with Hoechst 33342 represented in blue. The scale bar is 20 µm.
Figure 5.18 The overlay of the compressed z-stack images of the dendrimer uptake process in living SKMES-1 cells. The cells were incubated with medium containing 0.1% dendrimer (represent in green) and images were collected every 5 min before (A) and after adding the dendrimer (B-F). Images presented here are at time t= 5(B), 25(C), 45(D), 60(E) and 90(F) min of incubation period. Data were analysed over the time and plotted in G and the insert pictures show the positions of regions of interest (ROIs, white boxes) for data analysis. The mean fluorescent signal in each ROI was measured from projected Z-stack images taken at different time points and generalized by background subtraction. The dendrimer was found in the cytoplasmic compartment in the period of 5-25 min after exposed to the dendrimer (ROI 9 and 10). The concentration of the dendrimer declined after 26 min whereas the relative fluorescent intensity of the dendrimer inside the nucleus was detected (ROI 5-8). The cell nuclei were stained with Hoechst 33342 represented in blue. The scale bar is 18.14 μm.
For the dendriplexes the diffusion coefficients were expected to be even lower than that of the parent dendrimer. In the dynamic uptake study (Figure 5.19) the dendriplexes were found only in the cytoplasmic compartment throughout the stack images (A-D) after 2h of incubation. Although dendriplexes were, first, found attached to the cell membrane after 5 min of incubation, they were found to remain in the cytosol until the end of experiment (A-D). This was confirmed by the quantification of the fluorescent signal of the dendriplexes as plotted in Figure 5.19E (ROI 2-3, 7, 9, 12-13). There is a devoid of the fluorescent signal of the dendriplexes in the nuclear compartment throughout 2 h suggesting a longer $t_L$ value. In practical terms, the change in cell morphology was found in our live cell imaging system in the cell in absence of the dendrimer. From optimization and system validation the experiment should not be carried on for more than 2h in this system, although cells were aspirated and the temperature was controlled. Instead, the experiment was carried out using living cells incubated with the dendrimer at longer time points in the incubator and the cells were taken to view at the end of each incubation time point. The results are shown in Figure 5.20. The figures represent merge z-stack images (merge) and it transmission images (tran) of the cells which their nuclear and cell membrane are allocated by Hoechst33342 (blue) and WGA-Alexa Fluor® 595 conjugate (red), respectively. The dendriplexes were found to be taken up into nuclear compartment after 2.5h (G) of incubation and the uptake was seem to be widespread in nearly every cell after 3h (H-I). It is clear that uptake of the dendriplexes is time-dependent and that the dendriplexes take longer time to be taken up into nucleus. This was confirmed by the diffusion coefficient of the dendriplexes which is lower than that of the parent dendrimer in the cell cytoplasm, as shown in Table 5.1

From Figure 5.20 the lag time ($t_L$) here was found to be between 2.5-3 h (n=5), the diffusion coefficient of the dendriplexes in cytoplasm ($D_{cyto}$) was calculated to be 2.36 ($\pm0.34$ (SD)) $\times 10^{-11}$ cm$^2$/s (2.36$x10^{-3}$ μm$^2$/s) and the diffusion coefficients ($D_{cyto}$) relative to that in water (4.53 $\times 10^{-8}$ cm$^2$/s$^{-1}$ ($D_{water}$)) was 5.21 ($\pm0.75$) $\times 10^{-4}$. By comparison with the dendrimer $D_{cyto}$ of the dendriplexes relative to that value of the dendrimer in the Caco-2 cells was found to be 0.24. This means that the macromolecular crowding and obstacles in the cytoplasm confer an intimidating
barrier to dendrimer and dendriplexes transport. This effect is more pronounced when
the diameter of the compound is increased. The cytoplasm hinders the mobility of the
dendriplexes by 2000 times compared to that in water. This might be explained by the
interaction between the cationic dendriplexes and the cell organelles which impede the
mobility of the dendriplexes apart from effect of particles size, sieving effect and
obstruction of the crowded condition of the cells.

Table 5.1 Diffusion coefficients and relative diffusion coefficients of the parent dendrimers
and the dendriplexes in cell cytoplasm compared to other media

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Diffusion coefficients in various media (cm²/s)</th>
<th>Relative diffusion coefficients (cm²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>water</td>
<td>Actin gel</td>
</tr>
<tr>
<td>Dendrimer (6.5 nm)</td>
<td>79.54x10^{-8}</td>
<td>23.75x10^{-8}</td>
</tr>
<tr>
<td>Dendriplexes (107.3 nm)</td>
<td>4.53x10^{-8}</td>
<td>-</td>
</tr>
</tbody>
</table>

The differences are caused by two main factors: the physicochemical properties (size,
charge, mass) of the species and the uptake processes. The hydrodynamic diameter
and zeta potential of the dendriplexes were, respectively, 107.3 nm and 39.80 mV
compared to values for the parent dendrimer of 6.5 nm and 47.27 mV. The positive
surface charge of both compounds facilitates electrostatic attraction with the cell
membrane and they can be taken up by endocytosis. Concomitantly, the dendrimer
may be taken up by transient nanopores which can be created after adherence to the
cell membrane allowing also the passive diffusion resulting in the shorter uptake
process (Hong et al. 2004;Hong et al. 2006;Mecke et al. 2005c;Mecke et al.
2005a;Mecke et al. 2005b). Recently, polylysine has been found to initiate nanopores
in cell membrane with diameter of 1-10 nm (Hong et al. 2006). The larger size of the
dendriplexes would attenuate their diffusion inside cytoplasm.
Figure 5.19  The overlay of the compressed z-stack images of the dendriplex uptake process in living Caco-2 cells, the cells were incubated with complete medium containing dendriplexes (represent in green) and images were collected every 5 min before (A) and after adding the dendrimer (B-D). Images present here are at time t = 5(B), 30(C) and 60(D) min of incubation period. Data were analysed and plotted in E and the insert pictures show the positions of regions of interest (ROIs, white boxes) for data analysis in both channels. The mean fluorescent signal in each ROI was measured from projected Z-stack images taken at different time points and generalized by background subtraction. The dendriplexes were found to be taken up into the cytoplasmic compartment (ROI 2-3, 7, 9, 12-13) but there is no uptake of the dendriplexes into the nuclear compartment within 2h of incubation (ROI 4, 8, 10).
Figure 5.20 The time dependent uptake of the dendriplexes (green) in Caco-2 cells: the cells were incubated with complete medium containing dendriplexes at a final concentration of 0.67 μg/ml of LDNA for 0(F), 2.5(G), 3(H) and 3.5(I) h. The cells were rinsed with PBS and the fresh medium was added before the cells were observed under confocal microscopy. Cell nucleus and cell membrane were stained with Hoechst 33342 (blue) and the WGA-Alexa Fluor® 595 conjugate (red). The dendriplexes were found to be taken up into nucleus after 2.5 h. The diffusion coefficient of the dendrimer is calculated to be 2.36 (±0.34 (SD)) x10^{-11} cm^2/s.
In comparison to the dendrimer the attenuation of the diffusion coefficient of the dendriplexes is 4 fold. This complies with the changes in diffusion coefficient of the diffusant in aqueous solution when its radius is increased from 6.5 to 107 nm (\(D_{\text{water}}\) of dendrimer and dendriplexes are 79.54 \(x 10^{-8}\) and 4.53 \(x 10^{-8}\) \(\text{cm}^2\text{s}^{-1}\)) according to Stokes-Einstein equation. The diffusion coefficient in water of the dendriplexes is 20 times lower than the dendrimer. Although the effect of particles size precisely controls the diffusion of the particle in bulk or aqueous solution, it was found to also play a similar role in the diffusion of particles within the cells as explained by the \(D_{\text{cyto}}\) of dendriplexes and dendrimer which reduced from 9.82 \(x 10^{-11}\) to 2.36 (±0.34 (SD)) \(x 10^{-11}\) \(\text{cm}^2\text{s}^{-1}\) (4 times reduction) when the dendrimer (6.5 nm in diameter) formed a complex with the DNA (107 nm in diameter).

In the cells such diffusion coefficients can be explained by the obstruction effect regarding sieving property (Janson et al. 1996; Luby-Phelps 2000; Luby-Phelps et al. 1986; Luby-Phelps 1993; Provance, Jr. et al. 1993) of cell cytoplasm whereas that may not found for the dendrimer. Janson and Luby-Phelps proposed the pore slit model having an average cut off radius between 15-50 nm. Following this model the diameter of the dendriplexes is beyond the maximum cut off size, and thus could not pass through the cellular meshwork which present as the slow mobility of the dendriplexes at the rim of plasma membrane and could not traverse the cytoplasm (Figure 5.19B-D).

The dendrimers and the dendriplexes traverse cell cytoplasm to the nucleus can be proposed as the model depicted in Figure 5.21. The 1000 and 2000 fold reduction in diffusion coefficient of the dendrimer and the dendriplexes in cell cytoplasm can be dissected into two main steps: diffusion within cell cytoplasm and passage through nuclear pore complex (NPC). The factors involved in the first step are the obstruction and exclusion effects regarding the molecular crowding and the sieving effect mentioned above. In the latter step is controlled by transport mechanism of NPC.

The cell cytoplasm may act as a meshwork structure providing a sieving effect with maximum cut off size at 50 nm in diameter (Figure 5.21A). Likewise a gel, meshwork
structure and crowd molecules exhibit as insoluble compartment in gel possessing volume in which the dendrimer and the dendriplexes can not diffuse (Figure 5.21B). Thus the dendrimer and the dendriplexes have to detour in the non-excluded aqueous volume, until they reach their destination. After endocytosis or passive diffusion through transient nanopores (1-4), the dendrimer (red arrow) and the dendriplexes (green arrow) have to diffuse toward the nuclear compartment. Some of the dendrimer may interact with cell organelles (6) and the interaction can be found after endosomal escape of the dendriplexes (6). Particle size and types of the carrier systems seem to play a decisive role in the diffusion of the particles in this first step.

After endosomal escape, the debate is still whether or not DNA disassembly from the dendrimer occurs and how the DNA reaches the nucleus. Knowledge of transport mechanisms through the nuclear pore complexes (NPC) might help to make this clear. A vertebrate somatic cell usually contains between 1000 and 10,000 of NPC (Burke 2006). Transport of the DNA through NPC is assumed to be the rate limiting step for gene therapy; this process is divided into two strategies: passive and active pathways (Figure 5.21C). The particles with diameters between 8-10 nm can be transported by passive diffusion whereas the particles with larger size and particles with molecular masses between 25 and 75 kDa (Lusk et al. 2007) employ active transport. Recent models of NPC have been proposed as a hydrogel (Frey et al. 2006;Elbaum 2006;Peters 2006), as seen in Figure 5.21C. The selectivity is supplied by the meshwork construction of the phenylalanine glycine (FG) motif and the non-selectivity or passive transport is acquired from the aqueous tube of the channel center, 8-10 nm in diameter. DNA and dendrimers may passively diffuse though this channel center (Figure 5.21C1-2), while the dendriplexes may have to interact as transport complexes in the perinuclear membrane. These transport complexes are searching for the FG motif in “threading area”, seen in Figure 5.21C1, and they will only selectively passage via FG active transport pathways through the NPC (Figure 5.21C3).
Figure 5.21 An uptake model for dendrimer and dendriplexes: A demonstrates the sieving effect of the cytoplasmic meshwork with a cut-off size of 100 nm in diameter. The crowded condition and the obstruction effect cause the slow diffusion of the dendrimer and dendriplexes after endocytosis (1-2) or passive diffusion (3) through transient nanoholes, 1-10 nm in diameter, illustrated in B. The dendrimer and dendriplexes have to detour in the aqueous compartment excluding from crowded volumes. Some of the dendrimer and dendriplexes may interact with cell organelles or cytoplasmic proteins (4). Passing through the NPC, the DNA released from endosome (5) may cross the NPC by passive diffusion (1). As small as 6.5 nm, the dendrimer can diffuse through central pore having cut-off size at 8-10 nm (6) whereas the larger of dendriplexes, 107 nm, may employ active transport (7) (Figure C 1-4 are modified from Peters 2006, other parts are original).
5.3.4.3 The influence of fluid flow on the adhering of dendrimer

The adhering of the dendrimer by the cells was found to be impeded when the medium was flowing. Using a flow rate at 2 ml/min (0.033 ml/s) the cells were exposed to the dendrimer within a static medium (no flow) for 5 min. A similar impeded trend was visualised; the dendrimer started to adhere or adsorb to the cells due to electrostatic interaction as can be seen from the graph Figure 5.22. The fluorescent signal of the dendrimer (green) adhering on to cells was found to be reduced by 25% which mean that the flow of the medium could removes 25% of the attached dendrimer on the cell membrane (Figure 5.22A). Under this constant flow rate, the force of the fluid flow which impinges on the cells, the wall shear stress ($\tau_w$) can be calculated by the Navier-Stokes equation

$$\tau_w = \mu \gamma = \frac{6\mu Q}{a^2b} \quad \text{(5.7)}$$

where $\tau_w$ is wall shear stress (dynes/cm$^2$), $\gamma$ is shear rate ($s^{-1}$), $\mu$ is apparent viscosity of media (poise) at 37°C, $a$ and $b$ are chamber height and width (cm), and $Q$ is volumetric flow rate (ml/s). The viscosity of the medium measured at 37°C was found to be 0.0081 poise, $a$ and $b$ were 0.2 and 0.3 cm. Hence, the wall shear stress ($\tau_w$) was found to be 13.37x10$^{-2}$ dynes/cm$^2$ (12.54x10$^{-7}$ Newtons/cm$^2$). At the longer incubation period the fluorescent intensity of the dendrimer was reduced and remained stable indicating that the force from fluid flow is higher than the electrostatic force between the dendrimer and cell membrane. This force could remove the attached dendrimers from the cell membrane and prohibit the new attachment of the dendrimers as the fluorescent signal remains stable. A higher percentage of the attached dendrimer, 62.5%, was removed from the cell membrane to the circulating medium in SKMES-1 cells (Figure 5.22B) whereas this effect could not be seen in REFS-2 cells (Figure 5.22C). The disparate percentages of the removed dendrimer, perhaps, depend on the difference in the electrostatic force between each cell type and the dendrimer. Since the charge of the dendrimer is stable, the variation is therefore due to the charge of the cell surface which varied by the composition of membrane proteins in each cell line.
Figure 5.22 The influence of fluid flow on dendrimer adhering on Caco-2(A), SKMES-1(B) and REFS-2 cells(C): the cells were allowed to stay in complete medium for 5 min at 37°C and 5% CO₂ before the dendrimers were added (P) into the medium to achieve final concentration of 0.1% (w/v). The cells were incubated for another 5 min and the pump was then turned on (R) to start the circulation of the medium through perfusion chamber with flow rate of 2 ml/min as depicted in Figure 5.5. The stack images were taken every 5 min before, and after adding the dendrimer for 1h. The results indicate that fluid flow can obstruct dendrimer uptake in Caco-2 and SKMES-1 cells (A-B) while it shows no effect in REFS-2 cells (C).
5.3.4.4 Uptake pathway of the dendrimer and the dendriplexes

The results from Figure 5.23 demonstrate that the uptake of the dendrimer by cells may be dependent on endocytic-mediated endocytosis. After 5 min of incubation the dendrimer was found taken up by endocytosis and it is more pronounced at the longer period of dendrimer exposure as can be seen in Figure 5.23C-D. This follows the results obtained by Jevprasesphant et al. (2004) which suggested that a cationic G3 PAMAM dendrimer was taken up through endocytosis in Caco-2 cells.

However it might seem to be too early to conclude that the dendrimer is taken up only by endocytosis because other possible mechanisms such as the passive diffusion have not been tested. For endocytosis the further investigate by using endocytosis inhibitor should also be done to confirm this obtained result.

Similarly the uptake of the dendriplexes by Caco-2 cell was found to be effected by endocytic-mediated endocytosis as illustrated in Figure 5.24. The dendriplexes were first attached at the cell membrane owing to electrostatic interaction. Mislick and Baldeschwieler (1996) explained that membrane-associated proteoglycans may present as a receptors for transfection by cationic dendriplexes and this results in the cationic-mediated endocytosis.

Interestingly, the transport of the dendriplexes was found to be slower than that was found in dendrimer. At the similar incubation period the dendrimer were found in nuclear compartment while the dendriplexes were only found in cell cytoplasm. The difference in uptake rate in the same cell line may regard to the difference in size, charge and subcellular transport. The further study on this is indispensable.
Figure 5.23  The uptake pathway of the dendrimer (green, Ch1) in Caco-2 cells: the cells were incubated with complete media containing DMEM supplemented with 1% (w/v) MEM, 10% (v/v) FBS, gentamicin (50 mg/ml) and 0.1% (w/v) of dendrimers for 10 min (B), 30 min (C), 1 h (D). At t=25 min before termination the Texas red transferrin (red, Ch2) was added into the medium. The cells were well rinsed and fixed with 4% paraformaldehyde before viewing under confocal microscopy. In the control (A), the cells were exposed to the complete medium without the dendrimer. The dendrimer is found to be taken up by endocytosis as shown by the colocalisation (yellow) in the merge channel. At longer incubation times the endocytic-mediated endocytosis is more evident.
Figure 5.24  The uptake study of the dendriplexes (green, Ch1) in Caco-2 cells: the cells were incubated with complete media containing DMEM supplemented with 1% (w/v) MEM, 10% (v/v) FBS, gentamicin (50 mg/ml) and dendriplexes for 15 min (B), 30 min (C), 1 h (D). At t=25 min before termination the Texas red transferrin (red, Ch2) was added into the medium. The cells were excessively rinsed and fixed with 4% paraformaldehyde before viewing under confocal microscopy. In control (A), the cells were exposed to the complete medium without the dendriplexes. The dendriplexes are likely to be taken up by endocytosis as shown by the colocalistion (yellow) in the merge channel. At longer incubation periods the endocytic-mediated endocytosis is more prominent.

5.4 Conclusion

The intrinsically fluorescent lysine-based polymer has been proved to be used as a nano-fluorescent probe for tracking translocation and uptake of dendrimer within the cells as it show efficient fluorescent signal to be detected by both spectrofluorimetry and confocal microscopy. However, to use the dendrimer in combination with other fluorescent dyes, the excitation/emission spectra of the dyes and the concentration of
the dendrimer are the vital parameters due to the broad emission and excitation of the dendrimer. From these works the use the red fluorescent dye, $\lambda_{\text{exc}}=596$ and $\lambda_{\text{emis}}=615$nm, (Figure 5.23) appears to be more compatible with the dendrimer than the blue fluorescent dyes (Figure 5.15). This suggests that the red or far-red fluorescent dyes should be the most recommended when the dendrimer is used in the system.

The simple method developed for the dynamic uptake study of this nano-fluorescent probe has also been proposed. From this model the diffusion coefficients of the dendrimer and the dendriplexes have been evaluated. The uptake of the dendrimer and the dendriplexes depend on cell types and incubation period. The dendrimer was taken up into nuclear compartment whereas the dendriplexes also are but take much longer time. One uptake mechanism investigated here was found to be due to endocytic-mediated endocytosis. Other uptake mechanisms have also proposed. A model of uptake pathway of the dendrimer and the dendriplexes from cytoplasm throughout the nuclear pore complexes (NPC) have been proposed here. Macromolecular crowding in the cytoplasm presents a significant barrier to diffusion of dendrimer and dendriplexes and this has been discussed here in terms of exclusion and obstruction effects. Finally the uptake of the dendrimer was found to be impeded by the influence of fluid medium flow.

The diffusion of the dendrimer and the dendriplexes within the cell nucleus may be expected to be dependent on similar key factors obtained inside the cell cytoplasm. Effects of particle size remain eminent follows by the charge of the particles. However one question has been raised here: whether or not the dendriplexes release their DNA before or after passing through the NPC. The transport of the parent dendrimer through cell nucleus is expected to be involved by passive diffusion through the NPC. In case the dendrimer forms complexes with other cellular proteins, uptake could depend on the particle size of the complexes which attributed to the transport of the dendriplexes. Further investigation to clarify such problem is needed for the better understanding to achieve successful gene delivery systems.
In conclusion an understanding of movement of delivery vectors within cells and in the nucleus is essential for the development of effective systems. Good models of the physical structure of cell interiors are required along with probes such as the one described here to advance the design of new delivery vectors.
Chapter VI

STUDIES OF THE INTERACTION BETWEEN ACTIN AND A CATIONIC LYSINE-BASED DENDRIMER

Summary
In this chapter a 6th generation poly-lysine dendrimer, (Gly)(Lys)$_{63}$(NH$_2$)$_{64}$, is used as a model of flexible cationic nanocarriers to investigate interactions with the actin cytoskeleton. Firstly, the actual structure of actin inside cells was investigated using an immunofluorescent technique. An in vitro actin gel was also prepared and the construction of actin filaments and bundles was studied. Dendrimer-actin complexes were prepared by adding dendrimers to an actin gel and observed under confocal microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The molecular weight of the complexes were then analysed by SDS-PAGE and the biological activity of the actin after complex formation were evaluated using Western blotting. The viscosity of the actin gel after interaction with the dendrimer was measured using a micro-viscometer. Finally the effect of the dendrimers on actin polymerisation was investigated using spectrofluorimetry.
6.1 Introduction

To date, an assortment of nanoparticulate drug carrier systems have been developed to achieve high drug efficiency or high efficiency of gene delivery. Most of those systems have been investigated for their delivery and targeting abilities in vitro and in vivo, but little is known about the interaction between those carriers and the intracellular matrix after entering into the target cells, knowledge of which is important in the understanding of effective gene carrier systems. An overview of the interaction between the dendrimers and different substances will be given here as well as the interaction of dendrimers and the intra-and extracellular matrix.

Polyamidoamine (PAMAM) dendrimers (Starburst®) and their derivatives are commercially available and they have been studied in respect of their interaction with various substances including drugs, surfactances, cell membranes, the extracellular matrix and the intracellular matrix to elucidate their potential performance in drug delivery and targeting. This type of dendrimer was found to act as a solubiliser of the hydrophobic drug, ibuprofen, by forming a complex with the drug molecules through electrostatic interaction between the amine group of the dendrimers and carboxyl group of ibuprofen (Milhem et al. 2000). Another aspect is that some dendrimers act as surface active agents; such dendrimers were capable of forming aggregates and adsorbing at the air-water interface causing a decrease of the surface tension of water (Esumi et al. 2002). The interaction between dendrimers and surfactant molecules also resulted in a decrease in the critical micelle concentration (CMC) of the surfactant, sodium dodacyl sulfate (SDS), due to both the electrostatic attraction and hydrophobic interactions in the PAMAM-SDS complex (Bakshi & Kaura 2005; Bakshi & Kaura 2004).

For targeting, bovine serum albumin (BSA) is the major soluble protein found abundantly in circulatory system. It plays an important role in drug transportation and distribution. Binding to BSA is a crucial step of drugs and carrier systems to be directed to their target organ. Therefore, the interaction between dendrimers and BSA has been intensively studied in order to understand the fate of the dendrimer/drugs after administration. Most of the results suggested that the interaction between the
dendrimers with different surface groups and BSA involved electrostatic attraction (Purohit et al. 2003; Chiba et al. 2003). However, there are three domains in BSA molecules which affect the total charge of BSA molecule and thus the interaction between the dendrimers and BSA is dependent on the surface group of the dendrimers and the pH of the medium (Pan et al. 2005; Klajnert et al. 2003).

The interaction between the dendrimers and cell membranes is the first stage in entry for cell targeting. Dendrimers have been found to be able to activate or inhibit acetylcholinesterase enzyme on the human erythrocyte membrane. Both direct interaction between dendrimer and the enzyme and indirect interaction via membrane modification are responsible for the changes in enzyme activity (Klajnert et al. 2004). Dendrimer biocides consisting of poly(propylene imine) bearing quaternary ammonium groups were recently found to interact with cell membrane of both Gram positive and Gram negative bacteria and to subsequently disrupt and disintegrate the cell membrane. This destruction process was concentration dependent. At low concentrations, the dendrimers started replacing the surface divalent ions and binding to the cell membrane due to electrostatic forces between the positive dendrimer and the negative phospholipid membrane. Consequently the permeability of the cell membrane was slightly altered as the surface charge was readily negated or reversed. On increasing the dendrimer concentration, the membrane proteins were denatured and the dendrimers began to penetrate the lipid bilayer thus enhancing membrane permeability. Leakage of potassium ions out of the bacterial cell could be found at this concentration of the dendrimer well known as “bacteriostatic effect”. The further introduction of dendrimers destabilises the membrane structure and causes membrane disruption, exhibiting bactericidal effect (Chen & Cooper 2002).

Similar results were obtained from different types of dendrimers which possess antimicrobial activity concluding as antimicrobial mechanism. First the dendrimer interacts with the polar lipid head group of membranes; second hydrophobic interactions occur between the intra-layer aliphatic chain of the membrane and dendrimer surface groups (Klajnert et al. 2006; Klajnert & Epand 2005; Gardikis et al. 2006). The length of the hydrophobic branch chain of the dendrimers signified the
degree of the perturbation in membrane integrity. Results from atomic force microscopy (AFM) of the interaction between PAMAM dendrimers and the model of lipid bilayer, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), illustrated that the dendrimers bearing carboxyl and amine surface groups could promptly originate pore, in the lipid bilayer, ranging between 15-40 nm in lipid bilayer. The mechanism corresponded to that previously mentioned (Mecke et al. 2004).

The efficiency of in vivo gene transfer is rarely predicted from in vitro results. It is normally lower than expectation derived from in vitro work (Egilmez et al. 1996). This is attributed to the biological barriers which do not appear in cell culture systems. Extracellular matrix consists of sulfated proteoglycans containing core protein covalently linked to one or more sulfated or carboxylic glycosaminoglycans (GAGs) such as hyaluronic acid, chondroitin sulfate and heparin sulfate (Ruponen et al. 2003). The interaction between the cationic carrier of DNA complexes and the extracellular matrix may relax and release DNA prior to entry the target cells (Xu & Szoka, Jr. 1996). The transfection efficiency of dendrimer-DNA complexes is inhibited by the interaction of GAGs and the complex (Ruponen et al. 1999). The interaction with the extra- and intracellular matrix may alter the physicochemical properties of the DNA complexes such as surface charge and size, and thus affect the mobility of the complex inside cells and tissue (Pitkanen et al. 2003).

The interaction between a linear biological polymer, hyaluronic acid (HA), and the star shaped polymer, PAMAM dendrimers, was studied using static light scattering. The results implied that the binding ability between both polymers was dependent on the number ratio of the amounts of the amine surface groups inside the dendrimers to the amounts of the carboxyl groups in HA (Imae et al. 2003). In dilute solution of the dendrimer, the interaction can be explained by a "non-binding model" which means that the HA will act as a wormlike molecule and will not interact with the dendrimer. At the higher number ratio, this non-binding model will be transferred to an "average binding model" in that the dendrimer will start binding to the HA and change its structure into a rodlike structure when the number ratio is equal to 5. This can be explained by a critical binding model. It was found that every 1.5 repeating unit of
HA chain was bound with one dendrimer molecule. The interaction is due to electrostatic attraction as well as the hydrogen bonding between the carboxyl groups in the HA and the amide and amino groups in the dendrimer (Imae et al. 2003).

Eukaryotic cells need a cytoskeleton to maintain their cell shape and internal pressure. The intracellular structure is embedded and surrounded by a cytoskeleton as a dynamic cytoplasmic matrix. The cytoskeleton can be divided into three main types: microtubules (25nm in diameter), actin filaments (10nm in diameter) and intermediate filaments (7nm in diameter) (Alberts et al. 2002). Actin filaments are the most common structures found in cytoplasm. They are found as gel-like structures in cell cortex near cell membrane and as stress fibers in the cytosol. Some of them are found as an actin meshwork and as tight parallel bundles inside lamellipodia and filopodia respectively which helps cell to attach on the surface and move forward. There are few studies on the interaction between nanocarrier systems and actin although this type of protein is dominantly found in cell cytoplasm. In case of the dendrimers, as far as we can determine none of the experiments has been focused on their static and dynamic interactions with the actin cytoskeleton. In the work described here a 6th generation poly-lysine dendrimer, (Gly)(Lys)₆₃(NH₂)₆₄, has been used as a model of cationic nanocarriers in their interaction with the actin cytoskeleton.

6.2 Materials and Methods

6.2.1 Materials

The synthesis detail of a 6th generation poly-lysine dendrimers, Gly-Lys₆₃(NH₂)₆₄, as well as detail of characterization and purification could be found in Chapter II. Human Caucasian colon adenocarcinoma (Caco-2) cells obtained from European Collection of Cell Cultures. Dulbecco’s modified eagle medium (DMEM), Foetal bovine serum (FBS), MEM non essential amino acids (MEM), Trypsin-EDTA, Hanks’ balanced salt solution and phosphate buffer saline without calcium and magnesium ions (D-PBS) were purchased from Gibco, UK. Rhodamine phalloidin, Biotin phalloidin and Streptavidin Alexa Fluor 488 conjugate and Citiflour® mounting medium were purchased from Molecular probes, Netherlands. 1,4-Dithio-DL-threitol
(DTT) was purchased from Fluka, Switzerland. Actin extracted from rabbit muscle was purchased from Sigma, UK. Alpha-Actinin and Actin polymerization kit: Pyrene labeled actin, General actin buffer, Actin polymerization buffer, Adenosine 5'-triphosphate disodium salt (ATP), Tris-HCl was purchased from Cytokeleton, USA. Acrylamide®, Bis-acrylamide®, Trisma® base, Sodiumlauryl sulfate (SDS), Sodium chloride, Potassium chloride, Calcium chloride, Magnesium chloride, Imidazole, Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), D(+)-Glucose, N,N,N',N' - Tetramethylethlenediamine (TEMED), Ammonium persulfate, Bromophenol blue, Glycine, Anti-actin from rabbit (primary antiboby), Anti-rabbit IgG peroxidase conjugate (secondary antibody), Methanol, 3’3’5’5-Tetramethyl benzidine (TMB) liquid substrate system and Wide molecular weight range (molecular marker) were purchased from Sigma, UK. Iimmobilon-P (transfer membrane) was purchased from Millipore, UK. Skim milk powder was obtained from Tesco, UK. Beta-mercaptoethanol was purchased from Biorad, UK. SimplyBlue™ SafeStain was purchased from Invitrogen, UK.

6.2.2 Methods

6.2.2.1 Dendrimer synthesis

The details of the synthesising procedure and purification of the 6th generation polylysine dendrimer (Gly-Lys63(NH2)64) are described in Chapter II.

6.2.2.2 The study of actin filament in Caco-2 cells

6.2.2.2.1 Growing Caco-2 cells and cell passaging

Human Caucasian colon adenocarcinoma (Caco-2) cells were used as the reference cell line for studying an actin network. Caco-2 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplement with 1% MEM non essential amino acids (MEM) and 10% foetal bovine serum (FBS) at 37 °C, 10% CO2, in 95% relative humidity. The cells were expanded in 75 cm² cell culture flasks, T-flasks (Figure 6.1) until reaching 75-90% confluence. To harvest the cells, the medium in T-flask was removed and the cells were washed with 10 ml Hanks's balanced salt solution in order to remove traces of serum which would inhibit the action of trypsin. The cells were
then detached from the flask by treatment with 5 ml trypsin-EDTA at 37 °C for up to 5 min. Four ml of trypsin-EDTA was removed and the trypsinization stopped by adding 9 ml of complete medium into the same flask. The cell suspension was mixed well. The cell suspension, 1 ml, was pipetted and added into a new T-flask containing 19 ml of complete medium. The new flask was kept inside the same conditions as mentioned above. The cells were routinely passaged again when they reached 75-90% confluence. The rest of the cell suspension was centrifuged at 2000 RPM for 5 min and was used for plating if the cell viability was satisfactory (see 6.2.2.2.2).

**Figure 6.1** A morphology of the normal Caco-2 cells expanded in tissue culture flask (T-75 flask) until they reached their 80% confluence, shown here viewed under a light inverted microscope: A-1 day; B-3 days; C-6 days.

6.2.2.2.2 Cell viability and seeding

After reaching 80% confluence, the cells were harvested, centrifuged and the cell pellets were resuspended in 1 ml of the complete medium. The cell viability was studied using the trypan blue method. This method is based on the principle that viable cells do not take up dyes such as trypan blue, whereas non-viable cells do. Trypan blue solution (0.4% w/v) was added to the cell suspension (1:1) and the mixture was filled in the chamber of a haemacytometer. The viable and non-viable cells were counted in a counting grid under light microscopy and cell viability (%) expressed as

\[
\text{% Viability} = \frac{\text{Viable cell counted}}{\text{Total cell counted}} \times 100
\]

(6.1)

If the viability is more than 95 %, the cells are seeded on polylysine-coated glass cover slips in a 12-well culture support at a density of about 1x10^4 cells/cm^2. The cells were fixed and stained by primary (see 6.2.2.2.3) and secondary antibody before
observation under confocal microscope (see 6.2.2.2.4). At the same time the numbers of cells per milliliter were calculated by using the equation 6.2.

\[
    \text{Cell/ml} = \frac{\text{The number of cells counted}}{\text{The number of squares counted}} \times 10^4 \times \text{Dilution factor} \quad (6.2)
\]

6.2.2.2.3. Cell fixing and actin staining

After seeding the cells for 24 h they were rinsed with PBS once and fixed with 4% paraformaldehyde on ice for 5 min. The cells were then washed with PBS and incubated with 50 nM ammonium chloride for 10 min to stop the action of paraformaldehyde before being permeabilised with PBS solution containing 0.1% (v/v) Triton X-100, 10% (v/v) FCS and 0.5% (w/v) Bovine serum albumin (BSA) for 5 min. The cells were washed with PBS and subsequently washed with PBS contained 10% (v/v) FCS and 0.5% (w/v) BSA (FCS/BSA/PBS). The cells were incubated with primary antibody, biotin phalloidin (Figure 6.2), for 1 h and rinsed with FCS/BSA/PBS before they were incubated with secondary antibody, streptavidin Alexa Fluor 488 conjugate, for 30 min in dark condition. The cells were washed twice with FCS/BSA/PBS and PBS before the cover slips were mounted with glycerol jelly on microscope slides.

![Cover slip with Caco-2 cells growing on cover slip with Parafilm](image)

**Figure 6.2** The cells growing on poly-lysine coated cover slip are incubated with primary (1°) antibody staining, biotin phalloidin, at room temperature. The first 50 µl of 1°antibody was placed on parafilm. Then the cover slip containing Caco-2 cells was placed inversely onto antibody with cells facing down (A). B is a cross section of picture A.
6.2.2.4. Fluorescent microscopy of actin in Caco-2 cells

The specimens obtained from the procedure in 6.2.2.3 were observed under confocal microscopy using a x63 optical lens and single track mode. The excitation wavelength and emission wavelength of Alexa Flour are 488 and 515 nm, respectively.

6.2.2.3 *In vitro* actin gel preparation

6.2.2.3.1 An *in vitro* actin gel preparation

Actin stock solution, 1.5 mg/ml (33 µl), was added into buffer G which contained 0.2 mM adenosine 5'-triphosphate (ATP), 0.5 mM dithiothreitol (DTT), 0.2 mM calcium chloride, 1 mM sodium azide and 2 mM Tris-HCl, pH 8.0. Actin was polymerized by mixing 9 volumes of actin stock solution and 1 volume of polymerization buffer (buffer P) which contained 500 mM potassium chloride, 10 mM magnesium chloride, 10 mM EGTA and 100 mM of imidazole, pH 7.0. The gel was allowed to polymerize at least 2 h at room temperature in a cover well image chamber gasket (Figure 6.3).

![Figure 6.3](image)

*Figure 6.3* The Actin gel was filled into the cover well image chamber. First the protective liner from the cover well gasket was peeled off (A). The gasket was placed on the coverslip with the open-chamber side up and the 6.6 µM rhodamine phalloidin, 10 µl, was pipetted into the well (B). Then the actin gel specimen was filled into the well (C) after the rhodamine phalloidin was allowed to dry for 20 min. Finally the microscope slide was placed over the adhesive surface of the gasket with the whole pressed on microscope slide to seal (D).

6.2.2.3.2 Fluorescent microscopy of actin gel

Confocal fluorescent microscopy was used to qualitatively detect the heterogeneity of actin filaments. Ten microlitres of 6.6 µM rhodamine phalloidin in methanol was
deposited on the bottom of the cover well chamber and allowed to dry for 20 min (Tseng & Wirtz 2001). The actin gel prepared as described in 6.2.2.3.1 was added into the chamber gasket with or without alpha-actinin. The specimen was observed under confocal microscopy. The maxima excitation and emission wavelength of rhodamine phalloidin are 554 and 573, respectively.

6.2.2.4 The study of the interaction between actin filaments and dendrimers

6.2.2.4.1 Confocal microscopy

Actin stock solution, 2.5 mg/ml (20µl) and 2.5mg/ml of alpha-actinin (5µl), were prepared in buffer G (see 6.2.2.3.1). Dendrimer solution was added to achieve desired concentrations of dendrimer. The mixture was mixed well. Actin was polymerized by mixing 9 volumes of actin stock solution and 1 volume of buffer P (see 6.2.2.3.1), and the actin filament was stained with 6.6 µM rhodamine phalloidin as previously described in 6.2.2.3.2. The gel was then allowed to polymerize and can be kept overnight at 4 °C in a cover well image chamber gasket. The chamber slide was viewed under confocal microscope in the next day. The excitation and emission wavelength used to detect the dendrimers were 488 and 517 nm.

6.2.2.4.2 Transmission electron microscopy (TEM)

The actin gel was prepared by the procedure explained in 6.2.2.4.1. After overnight incubation, a sample of actin gel containing dendrimer (10-20µl) was added onto the TEM grid and excess sample was removed carefully using filter paper at the edge of the grid. The sample was stained with 1% uranyl acetate. After the stain was removed, the grid was air-dried for 5-10 min. Photomicrographs were taken at magnification ranging from 56,000x to 309,000x at an operating voltage ranging from 80 to 120 kV by Philips CM 120 (Einhoven, Netherlands).

6.2.2.4.3 Scanning electron microscopy (SEM)

A sample of actin gel containing dendrimer (15-20µl) from 6.2.2.4.1 was adhered to the SEM stub (TAAB Labs Ltd) using double sided carbon impregnated discs (TAAB Labs Ltd). The sample was then coated with gold in an Emitech K550 Spotter coater
for 2 min at 20mA. Subsequently the sample was viewed and photographed under a SEM (FEI Philips XL30) at magnification ranging between 4,000x and 20,000x at an operating voltage ranging from 10 to 12 kV.

6.2.2.4.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight of the dendrimer-actin complexes obtained from 6.2.2.4.1 was determined using SDS-PAGE. The method with modification of that described by Laemmli (1970) was carried out on a vertical slab gel apparatus using 1.5 mm spacer (Figure 6.4). The separating gel of 10% acrylamide and the stacking gel of 5% acrylamide were used as the supporting media (Figure 6.4D-E). The gel compositions presented in Table 6.1 were prepared by the procedure as described in Appendix IV. Ammonium persulfate solution (APS), 0.1%, was finally added to polymerise the separating gel and the mixture was filled immediately into the glass-plate sandwich by using 10 ml syringe. Care must be taken as the air bubble should not appear inside the gel. The overlay solution (0.01% SDS) was then added on top of the separating gel. After complete polymerization, the overlay solution was removed. The stacking gel was formed following the protocol described in Appendix IV and the mixture was immediately filled into the glass-plate sandwich on top of the separating gel. The sample-well comb (Figure 6.4C) was inserted into the stacking gel to make space for the samples, in the “sample well” (Figure 6.4F).

After the stacking gel was completely polymerized, the comb was eradicated, and the whole unit of gel and the glass-plate sandwich were attached to the electrophoresis chamber. The running buffer, prepared by the procedures as described in Appendix IV, was placed into the chambers. The sample (10μl), obtained from 6.2.2.4.1, was mixed together with the sample buffer, 5 μl, which was added as a colour indicator (Appendix IV) before loading. Standard marker proteins (Wide molecular weight range, molecular, Sigma) and the rest of samples were loaded into the wells. Electrophoresis was carried out at room temperature using 104 mA (200V) for 45 min. The gel was removed from the glass sandwich and was placed in a staining solution, SimplyBlue™ SafeStain, for 30 min with gentle shaking. Excess stain was removed by destaining with several changes of double distilled deionised water until
the background was clear, this gel was called “electrophoretogram”. The protein bands on the electrophoretogram was observed and recorded by photography.

**Figure 6.4** Vertical slab gel apparatus (Bollag & Edelstein 1991) equips with supporting media (A), glass-plate sandwich (B), sample-well comb (C) and electrophoresis chamber (G-H). Supporting media consists of separating gel (D), 10% (w/v) acrylamide gel, and stacking gel (E), 5% (w/v) acrylamide gel in this study. The sample wells (F) were made in stacking gel using sample-well comb.

### Table 6.1 Composition of separating gel and stacking gel

<table>
<thead>
<tr>
<th>Number</th>
<th>Ingredients</th>
<th>Separating gel (10% Acrylamide)</th>
<th>Stacking gel (5% Acrylamide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>30% Acrylamide</td>
<td>3.325 ml</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>2.</td>
<td>Solution A</td>
<td>2.50 ml</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>Solution B</td>
<td>-</td>
<td>0.95 ml</td>
</tr>
<tr>
<td>4.</td>
<td>Distilled water</td>
<td>4.00 ml</td>
<td>5.00 ml</td>
</tr>
<tr>
<td>5.</td>
<td>10% SDS</td>
<td>0.10 ml</td>
<td>0.075 ml</td>
</tr>
<tr>
<td>6.</td>
<td>10% APS</td>
<td>0.10 ml</td>
<td>0.075 ml</td>
</tr>
<tr>
<td></td>
<td><strong>Total volume</strong></td>
<td><strong>10.025 ml</strong></td>
<td><strong>7.35 ml</strong></td>
</tr>
</tbody>
</table>
6.2.2.4.5 Western blot

The biological activity of actin after forming complexes with the dendrimer was evaluated using Western blotting. This technique allows one to detect a specific protein recognized by a specific antibody. The proteins from electrophoretogram are transferred to nitrocellulose membrane. The protein fixed to the membrane is specific bound with primary antibody raised against it. Then the secondary antibody, which may be conjugated to a different reporter enzyme or molecules such as alkaline phosphatase, peroxidase and biotin, binds to the primary antibody. The substrate is applied to the membrane resulting in a colour reaction.

The samples obtained from 6.2.2.4.1 were loaded and run as described previously in 6.2.2.4.4. The nitrocellulose membranes (Figure 6.5D) and 2 pieces of Whatman filter (Figure 6.5C and 6.5F) paper were cut to the size of the electrophoretogram (Figure 6.5E). Next the membrane were soaked in methanol for 15 sec and rinsed with distilled deionised water for 20 sec before it was equilibrated in blotting buffer prior to assembly. The filter papers were soaked in blotting transfer buffer (see Appendix V). Both of the Scotch-Brite™ pads (Figure 6.5B and 6.5G) supplied with electroblot apparatus (Figure 6.5), Mini Trans-Blot (Biorad, UK), were soaked in blotting transfer buffer. The cassette (Figure 6.5H) was open and the soaking materials were arranged by laying down one Scotch-Brite™ pad (Figure 6.5G) and the pad was covered with one sheet of filter paper (Figure 6.5F). The filter paper was then covered with the membrane (Figure 6.5D) and the membrane was subsequently covered with electrophoretogram (Figure 6.5E). Any air bubble formed between the membrane and the gel should be eradicated. The other piece of filter paper (Figure 6.5C) and Scotch-Brite™ pad (Figure 6.5B) were, respectively, placed on top of the gel or electrophoretogram prior to closing the cassette (Figure 6.5H). The cassette was placed inside the module (Figure 6.5K). The ice unit was assembled (Figure 6.5I) and the chamber (Figure 6.5J) was filled with blotting transfer buffer. The Western blot was performed at 350 mA (100V) for 1h. Then the membrane was removed from the cassette and rinsed with TBST buffer (see Appendix V) for 2 min. Immediately, the membrane was covered with blocking solution of 1% (w/v) skim
milk in TBST buffer (see Appendix V), for 1h at room temperature with gentle shaking and kept overnight at 4-8 °C.

The blocking buffer was removed on the next day by rinsing the membrane with TBST with gentle shaking and the rinsed TBST was replaced by the fresh one every 2 min four times. The membrane was then incubated with primary antibody at room temperature for 1h. The membrane was then rinsed four times with TBST as mentioned before. Then the membrane was incubated with secondary antibody for 30 min and washed as previously mentioned. The TMB substrate was added to cover the membrane and the membrane was kept in the dark condition for approximately 5 min. The reaction was stopped by rinsing the membrane with distilled deionised water several times. The photograph of the membrane was taken and recorded.

Figure 6.5  The Mini Trans-blot cell and its assembly
6.2.2.5 Effect of the dendrimers on actin polymerisation

The Actin Polymerisation Biochem Kit (Cytoskeleton, USA) allows one to carry out quantitative/qualitative studies effect of the dendrimer and other substances such as proteins and cell/tissue extracts on actin polymerization. This kit is based on the enhancement of the fluorescence signal of pyrene (N-(1-pyrene)iodoacetamide) probe which is conjugated to the 374-cysteine amino acid at the c-terminus of actin molecule during actin polymerization process (Kouyama & Mihashi 1981). Pyrene is a fluorescent probe which is sensitive to hydrophobic environment (Kalyanasundaram & Thomas 1977). Upon polymerisation, the environment of the label (pyrene) changes and causes the increase in fluorescence signal to 20 times its non-polymerisation state (G-actin). The increase in the fluorescent signal is explained by the changes in conformation of the actin molecule which results in the increase in hydrophobicity of local environment of the pyrene molecules.

In addition, actin filaments usually require ATP and divalent cations to stabilize their molecules. During the actin polymerization process, F-actin or actin filament is initiated involving five main factors: a high temperature (37°C), pH 5-6, the presence of divalent cations (e.g. Mg$^{2+}$), moderate ionic strength, and ATP (Carraway & Carraway 1992). Conversely, the actin monomer or G-actin, is promoted at low temperature (4°C) and pH 8-9 in the solution of low ionic strength, calcium ions and ADP (Carraway & Carraway 1992). The kit provides pyrene-labeled actin (1 mg/tube), general actin buffer (100 ml), actin polymerisation (4 ml) buffer, ATP (100 mM, 1ml) and Tris-HCl (100 mM) at pH7.5 (6 ml). Before the experiment was started the general actin buffer was resuspended in 100 ml of double distilled deionised water (<$10^{-14}$ ohm) and kept at 4°C until used. The actin that was labeled with pyrene (pyrene-labelled actin) was resuspended with 50 μl double distilled deionised water and an aliquot, 5μl, was placed in 1.5 ml microcentrifuge tube that was immediately kept in liquid nitrogen until used. Actin polymerization buffer was resuspended with 1.8 ml of 100nM Tris-HCl, pH 7.5 and readily aliquot 200 μl in small Eppendorf tube before it was kept at -70 °C. G-buffer (Actin monomer buffer) was always freshly prepared prior to the start of the experiment by mixing 2 μl of ATP with 1ml of general actin buffer and kept on ice until used.
6.2.2.5.1 System validation

The measuring system was validated by measuring the fluorescent intensity of actin monomer solution and the polymerized actin (F actin solution). The fluorescent intensity of F actin should be 3 to 4 fold greater than that of the monomer. First actin monomer solution was prepared by adding 225 μl of G-buffer into 5 μl of the aliquot pyrene-labelled actin to achieve the final concentration of 0.4 mg/ml pyrene-labelled actin in the medium containing 0.2 mM CaCl₂, 5mM Tris-HCl, pH 8.0 and 0.2mM ATP. The monomer solution was stand on ice during the F-actin was prepared. Second the F-actin, polymerized actin was prepared by adding 225 μl of G-buffer into 5 μl of the aliquot pyrene-labelled actin. The solution was mixed well and the 25 μl of actin polymerization was also added with pipette up and down. This polymerization was then kept at room temperature (25°C) for exactly 1 h. Finally, the fluorescent intensity of both actin monomer and F-actin solutions was measured in quart cuvette by spectrofluorometer (Luminescence Spectrometer LS 50B, Perkin Elmer). All other solutions were kept on ice during the experiment. The excitation was set at 365 nm with 10 nm bandwidth and the emission was set to scan mode (385-420) with 10 nm bandwidth.

6.2.2.5.2 Effect of dendrimer concentrations on actin polymerization

The actin monomer was prepared as previously described in 6.2.2.5.1 and kept on ice. The sample was transferred into the cuvette and the intensity of the pyrene-labelled actin monomer was measured every 45 sec for 10 min using time drive mode. After 10 min various concentrations (0.01-1,000 μg/ml) of the dendrimers were added while the fluorescent intensity was continuously measured for another 10 min. Then the polymerization buffer, 25 μl, was added into the cuvette and the fluorescent intensity was measured for 100 min. The excitation was set at 365 nm with 10 nm bandwidth and the emission was set at 407 nm with 10 nm bandwidth.

6.2.2.5.3 Effect of dendrimer concentrations on the viscosity of actin gel

The actin monomer solution was prepared as previously mention in 6.2.2.5.1 and various concentrations of the dendrimer suspension was added into actin monomer
solution and the solution was mixed well and left to stand at room temperature (25 °C) for 10 min. The actin polymerization buffer was added to polymerised the actin and the solution was kept at room temperature (25 °C) for exactly 1 h before their viscosities were measured using an Anton Paar automated micro viscometer (Anton Paar Gmb, Austria) at 25 °C.

6.2.2.5.4 Effect of dendrimer surface charge on actin polymerization

The actin monomer was prepared as previously described in 6.2.2.5.1 and kept on ice. The sample was transferred into the cuvette and the intensity of the pyrene-labeled actin monomer was measured every 3 sec for 10 min using time drive mode. After 10 min various types of the dendrimers and surfactant molecules were added while the fluorescent intensity was continuously measuring every for another 10 min. Then the polymerization buffer, 25 μl, was added into the cuvette and the fluorescent intensity was measured for 100 min. The excitation was set at 365 nm with 10 nm bandwidth, and the emission was set at 407 nm with 10 nm bandwidth.

6.3 Results and discussion

6.3.1 The study of actin filament in Caco-2 Cells

The actin filaments inside Caco-2 cells labeled with phalloidin immunofluorescence, Alexa Fluor 488 (Ex 488/Em 518), are illustrated in Figure 6.6. The normal structures of actin bundles inside Caco-2 cells are presented in Figure 6.6A. Actin filaments were abundantly found near the cell membrane and some were found as stress fibers in the cytosol (Figure 6.6B-F). The cell cortex or terminal web where actin and actin binding protein form gel-like networks is demonstrated in Figure 6.6B and E. In Figure 6.6D a conjectural line of the cell membrane observed under transmission light microscopy has been drawn in order to provide more detail on cell structure. The structure of actin filaments in crawling cells are shown in D and E (Figure 6.6). It was found that actin filaments arrange themselves as an actin meshwork and a tight parallel bundle inside lamellipodia (eg. in microvilli) (Figure 6.6D) and filopodia (Figure 6.6E), respectively, to help cells to attach to the surface and move forward. Not only has actin played an important role in the intracellular matrix but it has also
involved the extracellular matrix, as can be seen from Figure 6.6F where the filaments in each cell were indirectly connected to those of its neighbours by anchoring junction or adhesion belts. These results correspond to cellular functions of actin which are essential for cell survival: cell motility (Rafelski & Theriot 2004; Carlier & Pantaloni 1997), maintenance of cell shape (Ingber 2003a; Ingber 2003b), cell attachment (McGarry & Prendergast 2004), anchorage of cell organelles, maintenance of cytoplasmic viscosity and regurate endocytosis (Hamm-Alvarez 1998; Bershadsky & Vasiliev 2005; da Costa et al. 2003). In addition, there is evidence of actin coupled to the lipid bilayer of the cell membrane increasing their mechanical stability. This stability is high enough to resist osmotic pressures of up to 1000 bar and to maintain the cell structure (Käs et al. 1997).

6.3.2 The study of an in vivo actin gel preparation

The structure of actin gel, 1 mg/ml (25 μM), is illustrated in Figure 6.7. It is noticed that the solution of actin which contained G-actin became more viscous when the polymerizing buffer was added. This might be because of G-actin was transformed to F-actin (actin filaments) or actin polymerisation was taking place. Generally, actin polymerisation happens in buffers containing moderate ionic strength and a slightly acidic pH. The presence of ATP and Mg\(^{2+}\) stabilized the polymerisation process as the self-assembly or disassembly of actin subunits relies on nucleotide hydrolysis (Carraway & Carraway 1992). Apart from those conditions, temperature also has a pronounced effect on actin polymerisation. It was found that actin was not polymerized at 4 °C although a polymerising buffer was added. Actin polymerization was, however, found to take place at room temperature. Yet the confocal microscopy results clearly did not provide any detailed structure of the actin filaments. This is because of the limitation of confocal microscopy, as with an optical lens x63 the minimum size of object which can be visualised is around 300-500 nm. The actual structure of the actin filament is 5-10 nm in diameter, much lower than this limit.
Note: see caption in the next page*
Figure 6.7 Immunofluorescence micrographs shows actin self-assembly of actin gel, 1 mg/ml, in polymerising buffer. The actin filaments are shown in grey scale.

If we consider the actin bundle inside Caco-2 cells observed in section 6.3.1, the fact should not be ignored that actin filaments inside cell cytoplasm form complexes with various intracellular proteins, including actin binding proteins, in order to form the stiff actin filaments, actin bundles or actin protein complex as which easily can be seen under confocal microscopy. Therefore the actin bundling protein, α-actinin 1.25 μM, was added into G-actin solution and the protein complex was polymerized at room temperature with polymerising buffer. The specimen was analyzed under confocal microscopy and the results revealed in Figure 6.8 and 6.9. A similar trend was also found after the polymerising buffer was added into actin-α-actinin mixture. The mixture appeared as a clear solution and was found to be more viscous. It could entrap air bubbles.

Figure 6.6 Immunofluorescence micrographs of the network of actin filament inside Caco-2 cells observed by confocal microscopy (objective x 63/1.4 oil DIP at the excitation wavelength of 488 with 2 (A, F), 3.2(B), 2.7(C-D) and 2.8 (E) scan zoom); the general structure of actin network (green) of Caco-2 cells (A); the cell cortex and stress fiber (B); the arrangement of actin filaments in lamellipodia (C-D) and filopodia (E); and the adhesion belt (F)
**Figure 6.8A** shows the structure of a gel of actin and α-actinin protein complex in a molar ratio of 25: 2.5 μM, respectively. Alpha-actinin coupled actin filaments and allowed us to see actin bundles under the microscope. By enhancing magnification, the length and diameter of actin bundles were measured (Figure 6.8B-C) and ranged from 5 to 100 μm with diameters of 0.33-0.90 μm. The short filaments were found moving in random Brownian motion. Interestingly the actin-binding protein promoted bundling and cross-linking of actin filaments. This is in good agreement with results obtained by Niederman (1983). There was also perpendicular alignment of actin filaments. Two types of filament intersections, either X- or Y-shaped, are indicated with the yellow arrow and a red arrow respectively in Figure 6.8. The Y-shaped intersection is seemed to be the most general one.

The z-stack images of the actin gel of thickness of 21 μm with the interval thickness of 1 μm were also taken and are presented in Figure 6.9. From this stack the three dimensional structures of actin gel were constructed and illustrated in Figure 6.10. Some of the filaments may be thought of as a two-stranded helix (white arrow) in the three dimensional (3D) structure of actin gel with a twist repeating every 45 μm. These results implied that there is a resemblance between an *in vitro* actin gel and the actin assembly inside the cytosol, for example, the Y-shaped intersection which can usually be seen in the cell cortex and lamellipodia (Alberts *et al.* 2002; Alberts *et al.* 2004). However, the concentrations of actin, 1 mg/ml (15 μM), and actin binding protein, 0.125 mg/ml (1.25 μM), used here were very low compared to cellular concentrations of actin, 10-20 mg/ml, and various types of actin binding proteins.
Figure 6.8 Fluorescent micrographs of actin gels comprised of actin-α-actinin protein complex, in a molar ratio of 25:2.5 μM in actin polymerising buffer. F-actin (A) was labeled with rhodamine phalloidin (grey scale) and observed under confocal microscopy (objective x 63/1.4 oil DIP at an excitation wavelength of 554 with 2.3 scan zoom). The length and diameter of actin bundles were ranged from 5 to 100 μm with diameters of 0.33-0.90 μm. There was also perpendicular alignment of actin filaments. Two types of filament intersections, either X- or Y-shaped, are indicated. B and C show the high magnification images of actin bundles. Red and yellow arrows show the Y- and X-shaped cross-linking.
Figure 6.9 The z-stack images of actin gel labeled with rhodamine phalloidin (red) with an interval thickness of 1 μm depth from the top to bottom. The gel was formed using actin/α-actinin in a molar ratio of 25:1.25 μM in actin polymerising buffer and was observed under confocal microscopy (objective x 63/1.4 oil DIP at the excitation wavelength of 543 with 2.2 scan zoom)
Note: see caption on the next page*
Figure 6.10  Three dimensional (3D) structure of actin gel conducted by superimposing the stack images in Figure 6.9 together and displays at 0, 46, 92, 138, 184, 207, 276, 322 and 345 rotational angles from xy-plane, respectively. F-actin (A) was labeled with rhodamine phalloidin (red) and observed under confocal microscopy (objective x 63/1.4 oil DIP at an excitation wavelength of 543). Each angle shows the details of the cross-linking between the filaments. Two types of filament intersections, either X- or Y-shaped, are dominant in the gel. Turquoise and yellow arrows show the Y- and X- shaped cross-linking. Actin filaments were sometimes found as a two-stranded helix (white arrow) with a twist repeating every 45 μm.
6.3.3 The interaction between actin filaments and dendrimers

6.3.3.1 Confocal microscopy, TEM and SEM

It was not unexpected that the cationic dendrimer used in this work interacted with the actin bundle as is clear from both confocal microscopy (Figure 6.11) and TEM results (Figure 6.12). In Figure 6.11 the dendrimer (in green) interacts with the actin bundle (in red) as can be seen from the co-localisation (yellow-orange colour in merged picture) of both materials. The results from TEM (Figure 6.12) reinforced the view that there is an interaction between actin bundles and the dendrimer as the dendrimer were found attached to the actin polymer. Figure 6.12A demonstrates the actin bundle and the crosslinking of actin filaments (Figure 6.12B) in an actin gel prepared in vitro. After forming the complex with the dendrimer (Figure 6.12B), it was found that the dendrimers may promote actin bundling more than cross linking of actin as can be seen from Figure 6.12C-D. The numbers of actin bundles were found to be more frequent than that in control specimen. It was envisaged that the dendrimers were aggregated (Figure 6.12E) and attached onto the actin bundle (Figure 6.12C-D). The white precipitate of the complex could also be visualised after the dendrimers were added into the actin gel. The amount of the precipitation was concentration dependent. An increase in the concentration of the dendrimer resulted in the increase in the amount of the precipitation. This might be due to the electrostatic attraction between the positive charges of the amine groups at the surface of the dendrimers and the carboxyl group of actin molecules as well as some hydrophobic interaction. At high concentration of the dendrimer, 1-10 mg/ml, it may strongly interact and form insoluble complexes with actin.

Figure 6.13 shows the influence of dendrimers on actin gels obtained from SEM. The results are in accordance with the results obtained from TEM as the presence of the dendrimer resulted in the disappearance of the actin crosslink (Figure 6.13B) whereas the actin bundles were remain (Figure 6.13C). This implied that cationic dendrimer may be involved in actin polymerization process.
Figure 6.11 Confocal micrograph shows co-localisation of the dendrimers (green) and actin (red). Actin was labelled with rhodamine phalloidin (red) and observed under confocal microscopy (objective x 63/1.4 oil DIP at an excitation and emission wavelength of 554 and 573nm). The excitation and emission wavelength used to detect the dendrimers were 488 and 517 nm. The co-localisation in orange colour in the merged picture indicates that the dendrimer interacts with actin bundles. Actin gel was prepared with a final concentration of 1.5 μM as described in 6.2.2.4.1. Dendrimer solution was added to achieve a final concentration of 1% (w/v) before the gel was added into a cover well image chamber gasket.
Figure 6.12 Transmission electron micrographs of actin gel which illustrates actin bundles (A) and actin filaments (B) with 40-100 and 8 nm in diameter respectively; actin gel was prepared with final molar ratio of actin to α-actinin at concentration of 1.5: 1.25 μM as described in 6.2.2.4.1 and the dendrimer, 1 mg/ml, was finally added. Dendrimers are found attached to the actin bundle (C) and aggregation of the dendrimers, 25-30 nm in diameter, in actin gel (E). The scale bar is 100 nm.
Figure 6.13 Scanning electron micrographs of an *in vitro* actin gel (A) and the actin gel with incorporated dendrimers 1 (B) and 100 (C) µg/ml. Dendrimers exhibited biphasic effects on actin polymerisation in actin gel depending on its concentration. This corresponds to results illustrate in Figure 6.15. Actin filaments normally found in the gel are obliterated at low concentration (B), 1 µg/ml, owing to the inhibitory effect of the dendrimer on actin polymerisation. Whereas production of actin bundles is augmented, when the dendrimer concentration is increased (C), 100 µg/ml, due to an activation of actin polymerisation.
6.3.3.2 SDS-PAGE and Western bolt

The results from SDS-PAGE demonstrated that the molecular weight of the complex remained in the same range as the control which implied a physical interaction between the dendrimer and the actin monomer (Figure 6.14A). The bands of actin (43kDa) in lane number 2 and α-actinin (100 kDa) in lane number 3 were found to be in correspondence with the molecular marker (lane no. 1, Figure 6.14A) on the electrophoretogram. The proteins found in actin gel are illustrated in lane number 4. After polymerization, actin monomer is elongated and transformed into actin polymer (actin filaments) and the presence of the actin bundling protein, alpha-actinin, was also facilitate actin assembly. As a result the protein bands found here includes actin monomer and alpha-actinin, and actin filaments and actin bundle which were found at molecular weight higher than actin per se (lane no. 4). When the dendrimers were incorporated into the gel, a white precipitate was observed and the sample was then analysed by SDS-PAGE (lane no. 5). There was no protein band on this lane which implied that the dendrimers could precipitate the proteins inside the actin gel. The sample was then centrifuged and analysed both supernatant (lane no. 6) and precipitate (lane no. 7). The precipitate was redissolved in 10 % (w/v) SDS solution. As expected all proteins inside the gel were present in precipitate. The presence of SDS may release actin and alpha-actinin from the dendrimer, as the bands of both types protein were detectable. The band of the dendrimers with molecular weight of about 8.0 kDa was also found here (lane no. 7). The results suggested that the interaction between the dendrimers and actin mainly involves electrostatic forces between the positive surface charge of the dendrimers and the negative charge of actin. This physical interaction is revoked by adding anionic surfactant.

Western blotting was used to confirm the physical interaction between actin and the dendrimers. If there is chemical interaction between the actin and the dendrimer, the interaction may alter the conformation and structure of the protein causing the modification of their biological activity. Results indicated that actin protein both before (lane no. 5) and during interaction with the dendrimers gave the same response to the immunoblot (lane no. 8) which confirmed that the biological activity of the actin was still active in comparison to the control (lane no. 2).
Figure 6.14 SDS-PAGE (A) and Western blotting (B) of the actin gel and the gel after the dendrimers were added: A:1=marker, 2=actin (MW 43 kDa), 3=α-actinin (MW 100 kDa), 4=actin gel, 5=actin gel+DM 1, 6=supernatant part, 7=precipitate part, B:1=marker, 2=actin, 4=α-actinin, 5=actin gel, 6=actin gel+DM 1, 7=supernatant part, 8=precipitate part. Actin was the precipitated by dendrimers due to electrostatic interaction between positive amino groups of dendrimer and negative charges of actin, as all proteins in the gel (lane 4 and 5 in A and B) can not be detected (lane 5-6 in A and lane 6-7 in B). This complex is reversible by adding anionic surfactant to the complexes as all proteins in actin gel are again detected in both the electrophoretogram and the membrane (lane 7 and 8 in A and B).
6.3.4 Effect of dendrimers on actin polymerization

6.3.4.1 Effect of dendrimer concentration on actin polymerization

There are three phases for actin polymerization: a lag phase, an exponential phase and a plateau phase (Figure 6.15). Proteins which bind to actin will alter the time course of these phases. For example, the “nucleation protein” extracted from Acanthamoeba spp. can decrease the lag time (Carraway & Carraway 1992). Poly-lysine dendrimer used here can alter the actin polymerization process as can be seen from Figure 6.16. The results illustrate that dendrimers at low concentration, 0.01-1.0 μg/ml, may behave as “G-actin binding protein” (Lal & Korn 1985). This means that the dendrimer can bind with the actin monomer causing a reduction in the concentration of actin monomer in the system, and thereby lengthening both the lag phase and the exponential phase. At higher concentrations, 10μg/ml, the dendrimers accelerated the time course in the exponential phase by inducing the occurrence of actin fragments. The protein that induces such a phenomenon is called a “severing protein” (Cooper et al. 1983; Wegner & Savko 1982). Finally, if the dendrimers were further introduced into the system at 10-100 μg/ml they can accelerate the polymerization by shortening both the lag phase and exponential phase; such a protein is coined a “nucleating protein” (Frieden 1983; Tobacman & Korn 1983).

![Figure 6.15 Phases of actin polymerisation includes the “lag phase”, “growth phase” and “steady state phase”](image-url)
6.3.4.2 Effect of dendrimer concentration on the viscosity of actin gels

Falling (rolling) ball viscometry is based on the measurement of the speed of a small steel ball rolling down a glass capillary that contains a solution one wants to know the viscosity. The apparent viscosity of the actin solution could reflect the interaction between actin and actin-binding proteins (Figure 6.17) which in this case it will indicate the interaction between the dendrimer and actin. It may also reflect the actin concentration, number of actin filaments and length of the filaments. In this case dendrimer was previously evident that it interacts to actin (see result in 6.3.4.1). We carried out the viscosity measurements at different angles. Results show that there is no effect of the shear stress (alternated by changing the inclination angle of the capillary) on the viscosity determination of actin polymerisation (Figure 6.17) as can be seen that the similar trend was found in all angles (50°, 60° and 70°). The viscosity of water was used as control. The viscosity of actin gel, F-actin, is higher than that of water and actin monomer solution. The actin monomer has viscosity nearly similar to that of water. The viscosity of the gel increases as the concentration of the dendrimer is increased from 0.001 µg/ml to 10 µg/ml and thereafter decreases.
slightly when the concentration is increased to 1 mg/ml. The increase in the viscosity implies that the dendrimers may accelerate the polymerization of actin in comparison to F-actin.

Statistical analysis of mean viscosities using ANOVA with Post Hoc test (Tukey HSD) indicated that there is significant difference between the control and actin-dendrimer complexes at all concentrations of the dendrimer used (p<0.05, represent as *). Similar trend was notice for the G-actin sample. However none of significant difference was found between the control/G-actin and F-actin sample. Interestingly, there is a significant increase of the viscosity when the dendrimer at concentration ranging from 0.01 to 10μg/ml were added into the actin gels (*) compared to control, G-and F-actin. This implies that dendrimer may aid the polymerisation of actin.

![Figure 6.17](image)

**Figure 6.17** Effect of dendrimer concentrations on actin polymerization: actin gels were prepared and different concentrations of the dendrimer were incorporated. The polymerization buffer was added and the gels were incubated at room temperature (25°C) for exactly 1h. The microviscosities of the gels were measured at 50°, 60° and 70° degree by Anton Paar automated microviscometer at 25 °C. The viscosity of the actin gels with or without dendrimer is higher than that of actin monomer (G-actin) and water (control). No significant difference in viscosities of the actin gels is found at all ranges of dendrimer concentration added to gel. The data set was analysed using ANOVA with Post Hoc test and the significant differences (*) were found as described in text.

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2 The Automated Microviscometer (AMVn) is based on the “rolling ball” principal. The sample was introduced into a glass capillary in which a steel ball rolls. The viscous properties of the sample can be determined by measuring the rolling time of the ball. A change in the inclination angle of the capillary varies the shear stress. Viscosity is determined using the following equation \( \eta = K(u) \cdot t_0 \cdot (\rho_k - \rho_s) \) where \( \eta \) and \( K(u) \) are viscosity (mPa.s) and calibration constant of the measuring system (mPa.cm^2/g); \( t_0 \), \( \rho_k \) and \( \rho_s \) are rolling time (s), ball density (g/cm^3) and sample density (g/cm^3), respectively.
6.3.4.3 Effect of dendrimer surface on actin polymerisation

Cationic poly-lysine dendrimers (DM1), PAMAM dendrimers, a linear polymer of poly lysine (LYS) and carboxyfluorescein can accelerate actin polymerization (Figure 6.18). The poly-lysine dendrimers, PAMAM dendrimers and the linear polymer of poly lysine behaved as the nucleating protein which can decrease lag time of the polymerization process. Carboxyfluorescein (CF) acted as a severing protein as it reduces the exponential phase of the polymerization process. However, it was found that both DM and PAMAM dendrimers can reduce the fluorescent intensity of the plateau phase which suggests that some dendrimers may bind to actin monomer.

Figure 6.18 The effect of surface charge and type of polymer on actin polymerisation: poly-lysine dendrimer (DM1), PAMAM dendrimer, linear lysine (LinLys) and carboxyfluorescein (CF) are found to activate actin polymerisation in different mechanism. DM1 (red line), PAMAM (blue line) and LinLys (orange line) display as a nucleating protein by decreasing lag time of polymerisation. While CF (brown line) exhibits as a severing protein which can reduce an exponential phase of the polymerisation process.

To date there has been no study on the effect of dendrimers on actin polymerisation. However the interactions between the dendrimers and other systems have been studied. PAMAM dendrimers were found to interact with amyloid fibril formation.
due to the fact that they could interact with fibrils and block fibril growth, as well as interacting and breaking existing fibrils (Klajnert et al. 2006). The inhibition process is generation and concentration dependent. The amyloid fibril formation is composed of three phases as the actin polymerization and the dendrimers were found to reduce the slope of the elongation phase that is comparable to the exponential phase of actin polymerization (Klajnert et al. 2006). Another type of dendrimer is a polypropyleneimine octaamine dendrimer which has been employed as a crosslinker for collagen in corneal tissue engineering (Duan et al. 2007). The study suggested that this type of dendrimer is superior to other common collagen crosslinkers such as glutaraldehyde and EDC (Duan & Sheardown 2006).

6.4 Conclusion

Actin filaments are abundant as gel-like structures in the cell cortex near the cell membrane and as stress fibers in the cytosol. Some filaments have been found as an actin meshwork and a tight parallel bundle inside lamellipodia and filopodia, respectively, which helps the cell to adhere to a surface and migrate. This is in accordance with the function of the actin cell cytoskeleton which provides cell shape, cell anchoring, cell motility and control viscosity of the cytoplasm.

For an in vitro actin gel study, there are at least four important factors for actin polymerization: pH (which should be between 5 and 7), temperature (which should be from 20 to 37°C), ionic strength, energy (ATP is the preferable) and the presence of divalent cation (of which Mg$^{2+}$ is preferable). Alfa-actinin promoted actin bundling with the bundles ranging from 5 to 100 μm in length with 0.33-0.90 μm in diameter. The detail of the cross linking between F-actin and α-actinin and the three dimensional structure of the actin gel have shown here.

In this study the dendrimers possessing positive charge is clearly found to interact with both the actin monomer (G-actin) and the actin bundle resulting in increasing in the apparent viscosity of the actin gel. The dendrimer can form an insoluble complex with actin. This complex formation is reversible by adding a high concentration of anionic surfactant (≥ 10 % SDS solution) as evidenced in SDS-PAGE. The precipitate
of the complexes could be redissolved in 10 % (w/v) SDS solution. One may question that such reversible complexes perhaps intervene in SDS-PAGE results. However, the concentration of SDS used in the stacking gel, separating gel and running buffer were 0.1, 0.13 and 5% (w/v), respectively, which is still less than 10%. The complexes, therefore, remain in an insoluble form or precipitate form which can not be visualized by SDS-AGE, as can been seen, respectively, in lane number 6 and 7 of Figure 6.14A and Figure 6.14B. As a result, the SDS-PAGE results can be used to evidently support the physical interaction between the cationic dendrimer and actin. This was subsequently confirmed by results from Western blots. Results from Western blots indicated that the biological activity of the actin is still remained after forming complexes with the dendrimer.

Finally the cationic dendrimer (DM1) can inhibit or activate actin polymerization processes depending on their concentration, but at a concentration of ≥ 10 μg/ml, it was found to induce actin polymerization. Similarly, linear poly-lysine and PAMAM dendrimer can accelerate actin polymerization by decreasing the lag phase and thus they perform as a mechanism of nucleating proteins. Remarkably, the acceleration was also found in the case of carboxyfluorescein (CF) which possesses a negative charge. This may be explained by the molecular structure of actin monomer bearing both negative and positive subunits, although its total charge is negative. Consequently, there is a chance that the negative charge of the CF may be attracted to the positive subunit of actin molecule and thus form complexes which could, perhaps, accelerate actin polymerisation. Further study of this is needed. However, it is clear that the interaction studied here demonstrates that the movement of dendrimer in actin gels in vitro and possibly in vivo is complex. Conceivably the most important aspect in respect of living cells is the binding of the dendrimer to the actin filaments.
Chapter VII

CONCLUSIONS AND FUTURE PERSPECTIVES

7.1 Conclusions

This thesis has described the synthesis and certain applications of some lysine-based dendrimers with special regard to their cellular transport discussed from the point of view of their potential as a gene carrier system and as interesting systems in their own right. The possession of an intrinsic fluorescent property allows one of the dendrimers to serve as a nano-fluorescent cellular probe. Diffusibility of this dendrimer in various types of media and in cells provided diffusion coefficients which elucidated both \textit{in vitro} and \textit{in vivo} transports.

The synthesis of the intrinsically fluorescent polylysine dendrimer (DM1), (Gly)(Lys)$_{63}$(NH$_2$)$_{64}$, was achieved by both Fmoc-SPPS (30% overall yield) and Boc-SPPS (64% overall yield) with the "divergent" synthetic strategy. A comparison between the compound obtained from both methods after characterisation using NMR, MS and RP-HPLC indicated that the Boc-SPPS method resulted in a higher yield and purer product.

The diffusion phenomena of the dendrimer (6.5 nm (DM1)) and nanoparticles (48-495 nm polystyrene latex) in Newtonian and isotropic systems has obeyed the Stokes-Einstein relationship that is the diffusion coefficient (D) was inversely proportional to the particle radius and medium viscosity. The D values can usually be measured under
standard procedures by PCS and FRAP techniques. When the medium becomes more concentrated and more complex, for instance, 30-60% (v/v) glycerol solutions or HPMC gels, the diffusion values of bulk dendrimer and nanoparticles has become inaccurate using PCS, as the Brownian motion is limited and the initiation of thermodynamic nonideality with a temporal aggregation, flocculation and clustering. Under conditions beyond the PCS limits, the FRAP technique has been proved to work well as the diffusion in high concentration glycerol solutions and in heterogeneous media actin gels could be practically accessed.

An application of the lysine-based dendrimers as DNA carrier systems was investigated using DM1 \( (\text{Gly})(\text{Lys})_{63}(\text{NH}_2)_{64} \), DM2 \( ((\text{C}_{18})(\text{Lys})_7(\text{NH}_2)_8)\) and DM3 \( ((\text{C}_{18})(\text{Lys})_7(\text{NH}_2)_7(\text{RGD})_1)\). All types of dendrimers are capable of DNA condensation. Complete condensation was found at 5:1 (DM1) and 4:1 (DM2-3) molar charge ratios. By comparison with the spherically symmetrical dendrimer (DM1), the DNA was better condensed by partial dendrimers or dendrons (DM2) possibly due to the synergistic effect between electrostatic and hydrophobic interactions. Although the dendrimer is considered at high concentration to be toxic in Caco-2, HEK-293, SKMES-1, REFS-2 cell lines, at the dendrimer concentrations required to form the dendriplexes they show relatively low toxicity. The optimal level of the dendrimers was elucidated, and transfection of pDNA was enhanced by forming the dendriplexes with all these dendrimers. From confocal microscopy and a luciferase assay, the transfection efficiency of the dendrimers compared to a commercial transfecting agent (Lipofectamine-Plus™ (LPP)) could be placed in descending order: DM3>DM2>LPP>DM 1 >pDNA.

The DM1 dendrimer can be used as a nano-fluorescent probe for tracking translocation and uptake of dendrimer within the cells because it provides an efficient fluorescent signal detected by both spectrofluorimetry and confocal microscopy. However, in case of using the dendrimer with other fluorescent dyes the excitation/emission spectra of the dyes and the concentration used of the dendrimer become the crucial parameters which need to be considered due to the broad emission and excitation of the dendrimer. The results suggest that the red or far-red fluorescent
dyes should be more recommended than the blue fluorescent dyes when the dendrimer is used in the system. A simple method for studying the dynamic uptake of this fluorescent probe has been established and allowed the cytoplasmic diffusion coefficients to be calculated. Values were found in the range $5.99 \times 10^{-11}$ cm$^2$ s$^{-1}$ (in SK/MES-1 cells) to $9.82 \times 10^{-11}$ cm$^2$ s$^{-1}$ (in Caco-2 cells) for the dendrimer and $2.36 \times 10^{-11}$ cm$^2$ s$^{-1}$ (in Caco-2 cells) for the larger dendriplexes. The difference reflected variation in the intracellular architecture of the cell types and the effect of particle size. The dendrimer was readily taken up into the nuclear compartment of the cells whereas the dendriplexes required a much longer incubation period. The uptake of both dendrimer and dendriplexes is likely to be involved by nonspecific endocytosis and their uptake and translocation has been proposed. The uptake of the dendrimer could be restrained the influence of fluid flow.

The DM1 dendrimer clearly interacts electrostatically with actin filaments in vitro. Since the DM1 dendrimer has intrinsic fluorescence the interaction between the dendrimer and actin could be simply visualised under the fluorescent microscope and confirmed by the TEM, SEM and SDS-PAGE results. Insoluble complex formation between the dendrimer and actin was reversible when a high concentration of SDS is added, indicating an insignificant chemical interaction within the complex, subsequently reinforced by results from Western blots. In a biochemical assay, DM1 exhibited biphasic effects on the actin polymerisation process, depending on its concentrations. At low concentration the dendrimer behaves as a “G-binding actin protein”, retarding actin polymerisation whereas at high dendrimer concentrations (0.01-1 mg/ml) it serves as a “nucleating protein” accelerating the polymerisation.

1.2 Future perspective

Further potential investigations deriving from the results acquired in this work could include:

- Diffusion studies of the intrinsically fluorescent dendrimer and dendriplexes in other biological media (cystic fibrosis sputum, cellular extracts), tumors and tissue as well as in cell nuclei and cell cytoplasm using cell injection and
FRAP techniques. This may lead to a better understanding of the influence of cellular barriers on the design of carrier systems. In addition, this also allows the development of an *in vitro* model for particular system.

- A quantitative study of these dendriplexes in comparison to the commercial available dendrimer-based transfecting agents (Superfect<sup>TM</sup>, Priofect<sup>TM</sup> and Astramol<sup>TM</sup>) to compare efficacy.

- The cellular translocation of the dendrimers and dendriplexes needs to be clarified in order to be able to understand and engineer the system to achieve higher gene transfection.

- The biphasic effects of the dendrimer on actin polymerisation indicate that dendrimer may be capable of providing an antiangiogenic effect. A preliminary study using a simple angiogenesis assay may lead to the new application as antiangiogenic agent.

- It might be important to study the interaction between the dendrimer/dendriplexes and cell organelles which may provide the better understanding of cellular transport and cellular barriers leading to a better development of gene carrier systems.
APPENDIX I  Methods for cleavage dendrimer form resin

Method A: Standard HF cleavage

1. The dried resin, a Teflon-coated stirring bar and the scavenger were placed in a Teflon reaction vessel.
2. The cap was screwed on the reaction vessel and cooled in liquid nitrogen for at least 5-15 min before distilling HF
3. HF, 10 ml/gram of the resin, was distilled and the temperature was maintained at 0-5 °C by salted ice-bath for 90 min.
4. HF was drained out using liquid nitrogen and wait until the all HF was evaporated under reduced pressure. Once all HF was evaporated, the reaction vessel was kept under vacuum for another 30 min.
5. The peptide and the resin were precipitated with cold diethyl ether (8-10 volumes) added dropwise.
6. The peptide and the resin were removed by filtration under reduced pressure using a sintered glass filter (porosity no.3).
7. The peptide was isolated, dissolved by stirring in either water or the appropriate dilution of glacial acetic acid or trifluoroacetic acid and lyophilised.

Method B: Standard TFA cleavage

1. The dried resin was placed into the round bottom flask
2. The 95% TFA, 2.5% water, 2.5% TIS solution (25ml/g of resin) was added and the mixture was kept at room temp for 2.0 h with occasional stirring
3. The resin was removed by filtering the compound under reduced pressure and the resin was rinsed with TFA (x5/6.5 ml TFA)
4. Combining the filtrate and evaporate the TFA using rotary evaporator (boiling point of TFA is 72°C)
5. The cold diethylether was added dropwise to precipitate the peptide and left the reaction on ice or in fridge (4-8 °C) from1h to overnight.
6. Filters the peptide dendrimer through glass sintered filter (porosity no. 3) under gravity and wash the precipitated dendrimer further with cold ether
7. After ether precipitation add the water to the residue and transfer mixture to a separatory funnel. 5% of acetic acid may be necessary to aid dissolution
8. Shakes the stoppered funnel well. Releases the stopper and allows the two layers to separate by standing. Isolates the lower (aqueous) layer and repeats this step for 3 times
9. Removes the upper layer and store in a clean flask. Returns the combined aqueous extracts to separating funnel
10. Adding small amount of fresh diethyl ether and repeat the extraction process (x3), each time the ethereal layer was removed and returned the aqueous layer to separatory funnel.
11. The aqueous layer was collected in a clean flask and lyophilised.
APPENDIX II  Reagents for plasmid purification and agarose gel retardation

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<tr>
<td>Distilled water</td>
<td>800.0 ml</td>
</tr>
<tr>
<td>Adjust the pH to 7.0 with NaOH</td>
<td></td>
</tr>
<tr>
<td>Isopropanol</td>
<td>150.0 ml</td>
</tr>
<tr>
<td>Distilled water qs to</td>
<td>1000.0 ml</td>
</tr>
</tbody>
</table>

### TE buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trisma base</td>
<td>1.21 g</td>
</tr>
<tr>
<td>Na₂EDTA·2H₂O</td>
<td>0.37 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>800.0 ml</td>
</tr>
<tr>
<td>Adjust the pH to 8.0 with HCl</td>
<td></td>
</tr>
<tr>
<td>Distilled water qs to</td>
<td>1000.0 ml</td>
</tr>
</tbody>
</table>

### TBE buffer (10x)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trisma base</td>
<td>108.0 g</td>
</tr>
<tr>
<td>Boric acid</td>
<td>55.0 g</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>20.0 ml</td>
</tr>
<tr>
<td>Distilled water qs to</td>
<td>1000.0 ml</td>
</tr>
</tbody>
</table>

Dilute this TBE buffer to 1x every time before use.

---

**APPENDIX III**  TEM monoculture preparation procedures

**Reagents for osmium tetroxide fixative**

1. Prepared the following solutions
   - Solution A: 2.26% Sodium dihydrogen orthophosphate (11.3g in 500 ml distilled water)
   - Solution B: 2.52% Sodium hydroxide (12.6g in 500 ml distilled water)
   - Solution C: 5.4% Glucose (0.54g in 10 ml distilled water)
   - Solution D: 1g of Osmium tetroxide vial

2. Mixing 83 ml of solution A and 17 ml of solution B and the pH of the mixture was adjusted to 7.3.
3. Adding 10 ml of solution C in 90 ml of the mixture from no. 2
4. Adding the osmium tetroxide into fixative bottle and the solution from no. 3 was added into the bottle. Mixes slowly for 4 h and stores the fixative in fridge in tight closed container until use.
APPENDIX IV Reagents for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Solution A

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M HCl</td>
<td>48.0 ml</td>
</tr>
<tr>
<td>Trisma base</td>
<td>36.3 g</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.46 ml</td>
</tr>
<tr>
<td>Double distilled deionised water qs to</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

Solution B

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M HCl</td>
<td>48.0 ml</td>
</tr>
<tr>
<td>Trisma base</td>
<td>5.98 g</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.49 ml</td>
</tr>
<tr>
<td>Double distilled deionised water qs to</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

Solution C (30% Acrylamide)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>30.0 g</td>
</tr>
<tr>
<td>N, N’-methylene bisacrylamide</td>
<td>0.8 g</td>
</tr>
<tr>
<td>Double distilled deionised water qs to</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

Running buffer (5x)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trisma base</td>
<td>15.1 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>94.0 g</td>
</tr>
<tr>
<td>10% SDS</td>
<td>50.0 ml</td>
</tr>
<tr>
<td>Double distilled deionised water qs to</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

(All the above solutions should be kept in light-protecting container at 2-8°C)

Overlay solution (0.01% SDS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>40.0 µl</td>
</tr>
<tr>
<td>Double distilled deionised water</td>
<td>3.0 ml</td>
</tr>
</tbody>
</table>

10% SDS solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Double distilled deionised water</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>

Sample buffer (2x)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double distilled deionised water</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>1 M Tris</td>
<td>200.0 µl</td>
</tr>
<tr>
<td>Glycerol</td>
<td>100.0 ml</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>200.0 µl</td>
</tr>
<tr>
<td>10% SDS</td>
<td>400.0 µl</td>
</tr>
<tr>
<td>0.1% Bromophenol blue</td>
<td>100.0 µl</td>
</tr>
</tbody>
</table>

10% Ammonium persulfate solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium solution</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Double distilled deionised water</td>
<td>100.0 ml</td>
</tr>
</tbody>
</table>
APPENDIX V  Reagents for Western blotting

TBST buffer
- Tris CHI 7.9 g
- NaCl 8.8 g
- Distilled water 900.0 ml
- Tween 500.0 μl
- Adjust pH to 7.5 with 2N NaOH
- Distilled water qs to 1000.0 ml

Blocking solution
- Dried skimmed milk 1 g
- TBST 100 ml
- Stir for 30 min until completely dissolved

Western blotting transfer buffer (10x)
- Trisma base 30.0 g
- Glycine 144.0 g
- Distilled water qs to 1000.0 ml

Western blotting transfer Tris-glycine (working solution 1x)
- 10x Western transfer buffer 100.0 ml
- Methanol 200.0 ml
- Distilled water qs to 1000.0 ml

Primary antibody
- Anti-actin antibody stock solution 20.0 μl
- Blocking solution 20.0 ml

Secondary antibody
- Anti- Rabbit IgG-POD stock solution 2.0 μl
- Blocking solution 20.0 ml

(All the solutions should be kept in dark at 2-8°C for a day)
REFERENCES


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membranes. *Int.J.Pharm.*, 327, 145-152.


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LIST OF PUBLICATIONS

COMMUNICATIONS


ABSTRACTS


