STRUCTURAL STUDIES ON GLYCOSYLATED

POLYAMIDOAMINE DENDRIMERS



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This thesis describes research conducted in the School of Pharmacy, University of London between 2007 and 2010 under the supervision of Dr Mire Zloh, Prof Steve Brocchini and Prof Sunil Shaunak, at Imperial College. 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 the text herein and have clearly indicated by suitable citation any part of the dissertation that has already appeared in publication.

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<u>16.03.2011</u> Date

ABSTRACT

Polyamidoamine (PAMAM) dendrimers are hyper-branched molecules. Glucosamine conjugates of generation 3.5 PAMAM dendrimers have been reported to have immunomodulatory and antiangiogenic activity by preventing the LPS (lipopolyssacharide) triggered inflammatory response. These conjugates are prepared by a divergent synthetic route and the saccharide loading on dendrimer end groups has been estimated experimentally. The position and the distribution of the conjugated saccharides is varied and cannot be determined. Hence it is not possible to determine detailed structureactivity correlations.

Using computational strategies, the aim of these studies is to understand the structural features of glycosylated dendrimers that contribute to their activity and identify the interactions of these dendrimers with the biological target. The overall goal is to use the increased insight about the possible mode of action of the glucosamine PAMAM conjugates to aid the design of more potent dendrimer derivatives.

A method was first developed to allow the *in silico* generation of 3D models of the modified dendrimers. The electronic properties of these molecules were studied and the Frontier Molecular Orbital theory was used to understand the reactivity involved with saccharide loading and distribution on the modified dendrimer's surface. Conformational flexibility and the potential availability of the sugars for possible interactions with the biological target were then studied by the molecular dynamics of fully solvated molecules. Molecular properties were estimated for all generated structures. The representative conformations of flexible glycosylated dendrimers were used in rigid docking studies with the protein called MD-2, which is a component of the LPS recognition system. The docking studies were followed by dynamic simulations of the glycosylated dendrimer with MD-2. Based on these studies, a mechanism of action for glycosylated PAMAM dendrimers has been proposed where the dendrimer glucosamine conjugate interacts with MD-2 at the opening of the cavity and thus prevents LPS binding.

This work demonstrates how molecular modelling techniques can be used to elucidate the structural features of dendrimeric structures and their interaction with biological targets. In this specific case, the knowledge gained about the structural features of glycosylated dendrimers allowed the establishment of a set of criteria to distinguish between active and inactive dendrimers and enabled the rational design of biological active hybrid molecules. This methodology has the potential to be expanded to the development of novel polymeric molecules towards specific biological targets.

The important thing is not to stop questioning. Curiosity has its own reason for existing. Albert Einstein

To my parents and to my avó Maria

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LIST OF ABBREVIATIONS

2D	Two dimensions
3D	Three dimensions
μg	Microgram
μl	Micro litre
μΜ	Micromolar
°C	Degrees Celsius
Å	Angstrom
AM1	Austin methods 1
B3PYL	Becke 3-parameter
BIP	Bactericidal/permeability- increasing protein
CNDO	Complete neglect of differential overlap
COSY	Correlation spectroscopy
DAB	Diaminobutane
DAE	Diaminoethane
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-
	3-grabbing non-integrin
DEAE	Diethylamino ethanol
DEPT	Distortionless enhancement by polarization transfer
DFT	Density functional theory
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDC	1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionisation
ESP	Electrostatic surface potential
FAE	Follicle-associated epithelium
FFT	Fast fourier transform
FMOT	Frontier molecular orbital theory

fs	Femtosecond
g	Gram
g	Gravity
GB/SA	General Born/surface area
GUI	Graphical user interface
h	Hour
HF	Hartree-Fock
HMBC	Heteronuclear multiple-bond correlation
HMQC	Heteronuclear multiple-quantum correlation
НОМО	Highest occupied molecular orbital
HPRT	Hypoxanthine phosphoribosyltransferase
HS	Human serum
IC	Internal coordinates
INDO	Intermediate neglect of differential overlap
IL	Interleukin
IUPAC	International union of pure and applied chemistry
Kd	Dissociation constant
Kg	Kilogram
L	Litre
LD	Lethal dose
LBP	LPS binding protein
LPS	Lipopolysaccharide
LRR	Leucine rich repeats
LRRCT	Leucine rich residues C- terminal
LRRNT	Leucine rich residues N- terminal
LUMO	Lower unoccupied molecular orbital
MALDI	Matrix-assisted laser desorption/ionization
М	Molar
mg	Milligram
MIF	Molecular interaction fields
min	Minutes

MINDO	Modified intermediate neglect of differential overlap
MIP	Macrophage inflammatory protein
ml	Millilitre
mM	Millimolar
MS	Mass spectroscopy
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAMD	Nanoscale molecular dymamics
NDO	Neglect of differential overlap
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser enhancement spectroscopy
ns	Nanosecond
PAMAM	Polyamidoamine
PAMP	Pathogen-associated microbial patterns
PBS	Phosphate buffer solution
PBMN	Peripheral blood mononuclear cells
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDB	Protein data bank
PEG	Polyethylene glycol
PLL	Poly(L-lysine)
PM	Parameterisation model
PME	Particle mesh Ewald
PPI	Poly(propylene imine)
ppm	parts per million
ps	picoseconds
PSA	Polar surface area
PSF	Protein structure file
PSMA	Prostate specific membrane antigene
PW91	Perdew–Wang exchange and correlation functional
QM/MM	Quantum mechanics/molecular mechanics
R&D	Research and development

RMSD	Root mean square deviation
RNA	Ribonucleic acid
rpm	revolutions per minute
RPMI	Royal park memorial institute (culture medium)
RT-PCR	Real time-polymerase chain reaction
RSV	Human respiratory syncytial virus
SAR	Structure activity relationship
SASA	Solvent accessible surface area
SCF	Self consistent field
SPC	Simple point charge (water model)
STO-3G	Slater type orbitals represented by a combination of 3Gaussian functions
STO-3G TLR	Slater type orbitals represented by a combination of 3Gaussian functions Toll like receptor
STO-3G TLR TIR	Slater type orbitals represented by a combination of 3Gaussian functions Toll like receptor Toll/Il-1 receptor
STO-3G TLR TIR TMS	Slater type orbitals represented by a combination of 3Gaussian functions Toll like receptor Toll/Il-1 receptor Tetramethylsilane
STO-3G TLR TIR TMS TNFα	Slater type orbitals represented by a combination of 3Gaussian functions Toll like receptor Toll/Il-1 receptor Tetramethylsilane Tumour necrosis factor α
STO-3G TLR TIR TMS TNFα TOCSY	Slater type orbitals represented by a combination of 3Gaussian functions Toll like receptor Toll/Il-1 receptor Tetramethylsilane Tumour necrosis factor α Total correlation spectroscopy
STO-3G TLR TIR TMS TNFα TOCSY TOF	Slater type orbitals represented by a combination of 3Gaussian functions Toll like receptor Toll/Il-1 receptor Tetramethylsilane Tumour necrosis factor α Total correlation spectroscopy Time of flight
STO-3G TLR TIR TMS TNFα TOCSY TOF TS	Slater type orbitals represented by a combination of 3Gaussian functions Toll like receptor Toll/Il-1 receptor Tetramethylsilane Tumour necrosis factor α Total correlation spectroscopy Time of flight Transition state
STO-3G TLR TIR TMS TNFα TOCSY TOF TS VMD	Slater type orbitals represented by a combination of 3Gaussian functions Toll like receptor Toll/Il-1 receptor Tetramethylsilane Tumour necrosis factor α Total correlation spectroscopy Time of flight Transition state

CHAPTER I – GENERAL INTRODUCTION

Dendrimer glucosamine has recently been proposed as a novel drug for the treatment of the inflammatory response triggered by the mammalian innate immune response to infection and surgical trauma¹.

This project focuses on the structural features of glycosylated dendrimers that contribute to their chemical / biological activity and the identification of the interaction sites with the biological targets. To this end it is necessary to have an understanding of the mammalian innate immune system and the triggering and signalling systems involved, as well as an understanding of dendrimer structures and the methods of physically and chemically characterising them.

Innate Immune System

The immune system is capable of protecting a human host from external and internal threats, such as infections². It comprises a multifaceted group of molecules, cells and organs that serve to protect the host through complex interactions and mechanisms. The main challenge of the immune system is self/non-self discrimination³. It has two major components the non-specific or innate immune (first line of defence) system and the adaptive immune system (second line of defence that accounts for memory to re-exposure)⁴.

The main differences between the two systems are illustrated in Figure 1.1.

Innate Immune System

Adaptive Immune System

- Immediate response
- Non-specific
- No immunological memory
- Requires time for response
- Antigen-specific
- Immunological memory

Figure 1.1 Main differences between the innate and the adaptive immune systems.

The innate immune system can be divided into two branches, afferent and efferent⁵. The afferent or sensing branch is involved in perceiving an infection and the efferent on how to respond to this infection⁵. Each of these branches can be further divided into humoral and cellular, the components of each of these are shown in Figure 1.2.

	Afferent (sensing)	Efferent (effector)
Humoral	LPB, CD14, collectins, properdin, C3b, pentraxins	Cytokines, antimicrobial peptides, lyzozyme, BPI, complement, lactoferrin, acute phase reactants
Cellular	TLRs, CD14, Dectin-1, others	Antimicrobial peptides, proteases, lipases, glycosidases, hydroxyl radical, oxygen halides, singlet oxygen, nitric oxide, others

Figure 1.2 Innate immune system afferent and efferent branches and their components Adapted from Beutler, 2004.

The cellular component of the innate immune system is composed of different cell types, a) macrophages (derived from blood monocytes and capable of engulfing and killing pathogens), b) dendritic cells (very important for presenting the antigens to the

adaptive immune system), c) polynuclear phagocytes like eosinofils, basofils and neutrofils (all of major importance for containing the infection), and d) mast cells (important mediators of allergic responses)⁵. The cell based immune sensing is achieved by means of pattern recognition receptors, like the Toll like receptors (TLRs), which are capable of recognising pathogen-associated patterns (PAMPs), such as lipopolysaccharide (LPS)⁵. These sensing mechanisms activate pathways for the synthesis and secretion of molecules such as cytokines and chemokines (belonging to the humoral efferent component of the innate immune system) that mediate cell communication mechanisms throughout the body. The role and structure of both TLRs and LPS will be further discussed in the next sections.

The humoral component of the innate immune system can also be divided in afferent and efferent components (Figure 1.2). Among the afferent components are the mannosebinding protein, which recognises mannosyl residues on the microbial surface^{6, 7}, the LPS binding protein and CD14 which also acts as recognition molecules of bacterial determinants^{8, 9}. The efferent components include antimicrobial peptides^{5, 10}, lysozyme, lactoferrin¹¹ and the complement system^{12, 13}.

Toll-like Receptors

The Toll protein was first identified in Drosophila as a transmembrane receptor¹⁴. In mammals, homologs of the Toll protein have been found and given the name of Tolllike receptors (TLRs)^{15, 16}. These receptors are present in several immune cell types¹⁷ and they recognise and bind the pathogen-associated molecular patterns (PAMPs)¹⁸. The TLR family exhibits a conserved architecture, though different TLRs recognise different PAMPS (Table I).

Table 1.1 Different pathogen-associated patterns recognised by the TLR receptors family¹⁹⁻²¹.

Tall like recentors	Pathogen-Associated
Ton-like receptors	Patterns (PAMPs) recognised
TLR2 complex with	lineproteing or lineportides
TLR1 or TLR6	inpoproteins or inpopeptides
TLR3	viral double-stranded RNAs
TLR4	lipopolysaccharides
TLR5	bacterial flagellins
TLR7 or TLR8	single-stranded RNAs
TLR9	microbial DNAs

Structurally, TLRs are glycoproteins with an extracellular domain, a single transmembrane domain and an intracellular domain²⁰. The extracellular domain is responsible for the recognition of ligands and it consists of repeats of leucine rich repeats (LRR) modules²⁰. The intracellular domains of these receptors are Toll/Interleucine-1 receptor (TIR) domains²². It is believed that dimerisation of TLRs after ligand binding leads to a TIR-TIR interaction that is crucial for the activation of the signalling pathway²².

Around 20 modules of LRR are present in the extracellular domain of TLRs²³. Proteins with this configuration have a common horseshoe-like shape²⁰. The middle LRR module contains both conserved motifs and variable regions²³⁻²⁵. The conserved element 'LxxLxLxxN' forms parallel β -strands generating a concave surface on the LRR modules²³⁻²⁵. The variable regions of the LRR modules consist of helices and/or loops which form a convex surface²⁶.

The Toll-like receptor family presents one other typical feature, they require dimerisation for TIR interaction and intracellular cascade activation¹⁸, leading to M shaped dimers (Figure 1.3)²⁶ regardless of being heterodimers (TLR2-TLR1) or homodimers (TLR3-TLR3). Some TLR receptors require accessory proteins for dimerisation. These proteins are usually vital for the primary protein's function, the recognition of PAMPs and subsequent activation of the signalling pathways. The

accessory proteins might be required for folding, stabilisation or for direct interaction with the PAMPS. This is the case of TLR4 which requires the accessory protein MD-2 for function.



Figure 1.3 Toll-like receptor family dimmer formed upon ligand binding. A – TLR2– TLR6–Pam₂CSK₄ complex (3A79). B - TLR3–dsRNA complex (3CIY). C - proposed model of the TLR4–MD-2–LPS complex. Adapted from Jin and Lee, 2008^{26} .

Shigella and Shigellosis

Shigellae are gram-negative bacteria which belong to the Enterobacteriaceae family. These bacteria are facultative anaerobic bacilli. The first species discovered was *S. dysenteriae* type 1 in the 1896 by Kiyoshi Shiga and comes from the genus *Shigella*²⁷. There are four known Shigella species capable of causing disease, *S. dynsenteriae*, *S. flexinary*, *S. boydii* and *S. sonnei*. Each of these species is further subdivided into serotypes. These serotypes are defined based on the O antigen component of lipopolysaccharide, which can be found in the external membrane of the bacterial cell wall²⁸. The enteric bacteria from the genus *Shigella* are pathogenic and cause an invasive infection known as Shigellosis or bacillary dysentery²⁹. Shigellosis is a public health problem especially in developing countries, with low hygiene water supplies and affecting mainly children, increasing mortality³⁰.

Shigella species produce different types of toxins. Among these is included endotoxin LPS. However there are other toxins including Shiga protein which has neurotoxic, cytotoxic enterotoxin effects and there are several types of enterotoxins which depend on the species and type. These toxins can cause severe organ damage and lead to death³¹.

Disease prognosis is dependent upon the bacteria species and serotype, however the pathogenesis is related to a) its ability to cross the epithelium (e.g. follicle-associated epithelium, FAE)³²; b) its intrinsic invasive capability for epithelial cells³³; and c) the inflammatory response caused³⁴, which leads to a loss of barrier function within the intestine and in acute cases can lead to tissue necrosis and sepsis^{35, 36}.

Lipopolysaccharide (LPS)

Further explanation of LPS is necessary. LPS is an endotoxin found in the outer membrane of gram negative bacteria, like *Shigella* and *E. coli*. This component contributes to the membrane integrity and confers protection against chemical agents. Many studies involving LPS have been conducted with *E. coli*³⁷ since this organism is widely disseminated and is used in the production some protein based medicines.

LPS has been of great interest since the eighteenth century where the search began for a substance that led to fever and disease often when hygiene conditions were poor³⁸.

Relatively pure preparations of LPS were obtained only in the 1930's. Two decades later, the introduction of better extraction methods allowed more pure preparations to be obtained³⁸. The difficulty of performing structural studies with LPS is related to their amphipathic nature and their propensity to form aggregates³⁸. Although, the development of techniques such as mass spectrometry and NMR has contributed to the structural studies on these molecules, the interpretation of the spectra obtained by these techniques is still complicated due to the heterogeneity of these molecules. LPS molecules have a general architecture with a glycosidic core formed of monosaccharides, a lipid moiety also known as lipid A (endotoxin component) and O-antigen responsible for the diversity of LPS and the immunospecificity of bacteria²⁸ (Figure 1.4).



Figure 1.4 Schematic representation of the Gram-negative cell envelope and lipid A detailed structure³⁹.

The core of LPS molecules is highly diverse among different bacteria. It can be generally divided into enteric and non-enteric cores but there is further variability within each category³⁸. The enteric cores are usually composed of 8-12 sugar units, which are mostly branched. The last sugar, α -3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo), is conjugated to a glucosamine of the lipid A portion. This last unit can have one or two Kdo units bound to its C4 position³⁸. One other feature of these cores is the presence of L-glycero-D-*manno*-heptose residues which is often found as a substituent bound to the first Kdo^{40, 41}. This type of core is present in bacteria like *Shigella* and *E. coli*. Non-enteric cores are present in bacteria such as *Pseudomonas*³⁷, and *Francisella tularensis*⁴². The structural heterogeneity among these cores is very large³⁸. In the non enteric cores the Kdo unit is sometimes replaced or is bound together with Ko units (D-glycero-D-*talo*-oct-2-ulosonic acid)⁴³. The heterogeneity of bacterial cores can have its source in their incomplete biosynthesis or in the partial degradation after complete biosynthesis³⁷. However, many other factors may also contribute such as the growth medium and the phosphate stoichiometry³⁸.

The O-antigen is a polysaccharide chain that extends from the core out of the membrane. In pathogens this is portion of LPS in direct contact with the host²⁸. These chains can be linear or branched^{44, 45}. They can be highly variable defining the specificity of bacterial serotypes²⁸. The O-antigen is extremely immunogenic and contributes to the diversity and selection against specific antigen forms⁴⁶. Another contributing factor is that these O-antigen has also been thought to be responsible for selective advantage of various clones in their biological niches⁴⁶. The O antigen is also related to the virulence and pathogenecity of bacteria⁴⁸. Studies in *E. coli* and *Shigella flexneri* have shown that changes in these chains can alter the nature of pathogenicity and change virulence⁴⁹.

The lipid A of LPS is the endotoxin portion and is a glucosamine-based saccharolipid⁵⁰ with hydrophobic moiety. It typically is less struturally heterogeneous than the core and O-antigen³⁸. In general the lipid A and Kdo domains of LPS are required for bacterial growth^{51, 52}. Enterobacterial lipids A were the first to be studied and they follow a general architecture: positions 3 and 3' present ester-linked

bisphosphorylated β -(1 \rightarrow 6)-linked glucosamine disaccharide substituted with fatty acids and positions 2 and 2' present the same but connected through an amide-link⁵³ (Figure 1.4). Both positions 4 of GlcN^I and 6 of GlcN^{II} are free, though the glycosidic region is attached to position six⁵³.

Fatty acid chains in lipid A molecules usually have 10-16 carbon atoms. The length of these chains is thought to be important for the LPS toxicity since the most toxic LPS molecules tend to have C₁₂, C₁₂OH, C₁₄, and C₁₄OH fatty acid chains⁵³. The number of fatty acid groups is also a relevant feature since it is believed to have a direct effect on the toxicity of LPS^{53, 54}.

LPS and the Innate Immune System

As previously mentioned LPS is present in the external membrane of the cell wall of gram-negative bacteria. Its endotoxin component, lipid A is recognised by the TLR/MD-2 complex of mammals' immune cells. This is a part of the innate immune system³⁷. Low concentrations lipid A have beneficial effects on local infections⁵⁵, leading to the production of inflammation mediators like TNF- α and IL- $\beta^{56, 57}$, stimulating the production of tissue factors^{58, 59} and the activation of co-stimulatory molecules important for adaptive immunity^{60, 61}. However, when overproduced some of these molecules (cytokines and chemokines) have serious adverse effects on the body which lead to septic shock⁶².

The LPS recognition system in the immune system involves at least four different proteins: LPS binding protein, CD14, MD-2 and TLR4⁶³ (Figure 1.5). The structural features and interactions of this recognition system will be discussed in Chapter V.



Figure 1.5 Schematic representation of the LPS recognition system. Adapted from Miyake *et al*⁶⁴.

Dendrimers Structure and Applications

In 1985 Tomalia et al. reported the synthesis and characterisation of hyperbranched, polymer-like molecules which they called dendrimers, from the Greek "dendron" meaning tree⁶⁵. One other group independently reported the synthesis of similar molecules called arborols, from the Latin word for tree, "arbour"⁶⁶.

Dendrimers are spherical macromolecules with a three domain architecture, consisting of a core, multiple branches and numerous terminal end groups⁶⁷ (Figure 1.6).



Figure 1.6 Topology of dendrimers. Adapted from Klajnert et al.⁶⁸.

These molecules can be synthesised by either divergent or convergent methods⁶⁹. The divergent methods are better suited for large-scale production. The dendrimer synthesis occurs from the core where consecutive layers of monomer (branches) are added, each layer representing a generation (Figure 1.7 A). This method, however, has some drawbacks since incomplete and side reactions result in structural heterogeneity, especially as the dendrimer generations increase. There are also purification problems^{68, 70} when trying to separate dendrimers that differ only in a few bonds. To overcome these issues, convergent methods of synthesis were developed⁷¹. In these methods the structure is built from the outside of the sphere towards the dendrimer core. The monomers are attached to form the arms that are then linked to the core (Figure 1.7 B). Convergent methods, though solving the purification and side reaction problems of the divergent methods do not allow the synthesis of high generation dendrimers due to steric effects that are encountered when trying to bring the dendrimer fragments (i.e. dendrons) together to react with a reagent that forms the core⁶⁸.



Figure 1.7 Methods of dendrimer synthesis. A – divergent method; B – convergent method.

The first dendrimers to be synthesised were polyamidoamine (PAMAM) dendrimers also known as starburst dendrimers. They were synthesised by a divergent method⁶⁵. Tomalia et al. examined the synthesis of PAMAM dendrimers based on ammonia and EDTA as initiator cores. The branches were made using N-(2-aminoethyl) acrylamide that underwent Michael additions followed by amidation reactions with an alkylene diamine (a scheme of a generation two dendrimer is shown Figure 1.8).



Figure 1.8 Schematic representation of the formation of a generation 2 PAMAM dendrimer. Adapted from Tomalia *et al.*⁶⁵.

Each complete reaction creates a generation with an amide group with an amine at the end giving the molecule a cationic character at neutral pH (Figure 1.9 A). The number of terminal groups doubles with each increasing generation⁶⁸. The amine end groups can be allowed to react with carboxylic acid alkylating agents as a means to functionalise the end groups with carboxylic acids. If the reagents do not lead to branching there is no
increase in the number of end groups. In this case, the change from amine to carboxylic acid is termed a half generation for these dendrimers (Figure 1.9 B).



Figure 1.9 Amine and Carboxylic acid terminated PAMAM dendrimers. A – gen. 2 PAMAM dendrimer; B – gen. 1.5 PAMAM dendrimer.

The synthetic methods and structure of dendrimer molecules determine their properties and applications. Their intrinsic features like controlled molecular weight, controlled branching and versatility in the disposition of the terminal groups as well as the possibility to use a wide variety of cores and monomers⁶⁵, make the potential structures of these molecules almost limitless. Many applications have been examined including biomedical applications.

The development of characterisation techniques and the development of industrial and biomedical applications of dendrimer molecules have been extensive. Glycodendrimers and peptide dendrimers applications have been explored for the study and understanding of carbohydrate-protein and protein-protein interactions, particularly, for the interactions involving intercellular recognition events^{72, 73}. Peptide dendrimers have been studied as protein mimics, antiviral, anticancer agents and vaccines, among others^{74, 75}. Glycopeptides dendrimers applications have been further reviewed by

Niederhafner et al. for their role in immunotherapy and diagnosis of cancer and viral diseases⁷⁶.

The architectural features of these molecules along with the size, density and shape control as well as surface functionality have been compelling features that made them suitable for study in drug delivery. The use of dendrimers as nanodrugs and drug delivery agents have been reported, including encapsulation and conjugation of bioagents to their surface^{77,78,79}.

The use of a dendrimer as an encapsulating agent by the modification of a generation 5 poly- (propylene imine) (PPI-5) dendrimer with Boc-protected phenylalanine was investigated⁸⁰. A guest molecule (Bengal rose dye) was encapsulated into the Meijer dendrimer box (Dbox) during the modification process. Molecular dynamics techniques showed the dendrimer surface to be impermeable to encapsulated molecules, the dye molecules could not leave the interior even when excess was forced inside the Dbox⁸¹. The concept of dendrimers as "boxes" has been applied to the encapsulation of catalytic metal nanoparticles⁸² and lanthanide cations⁸³ and their relevance as drug delivery carriers has been widely explored⁸⁴.

Dendrimers can be synthesised with different environments throughout their structure. They can have a hydrophobic interior and hydrophilic branching and surface for example. Such dendrimers can form unimolecular micelles^{68, 85}. Anionic dendrimers have been shown to be able to bind different lipophilic probes such as diphenylhexatriene⁸³ and to trap hydrophobic drugs as indomethacin⁸⁶ as well as fluorescent molecules⁸⁷. These studies suggest that this type of dendrimer constitute good candidates for slow drug release^{77, 86}. The possibility of conjugating metal ions along with a targeting vector to the surface of a dendrimer to allow for site specific delivery has open the possibility to explore dendrimers as imaging agents^{88,78}.

In cancer, dendrimers have been studied both as potential diagnosis and therapeutic agents⁸⁹. Again drug encapsulation^{90, 91} and micelle⁹² systems have been evaluated in preclinical studies for anticancer therapies. Drug conjugates have also been described including several strategies with targeted drug delivery⁸⁹, for example, delivery systems for folic acid⁹³ and monoclonal antibodies⁹⁴ have been reported. A generation 5 PAMAM dendrimer conjugated with variable number of folic acid molecules has been

reported to increase binding avidity to folic acid receptor-over expressing cells as the number of molecules conjugated increases, however no improvements on the cell uptake rate were observed. The result of this study suggested the key event for tumour reduction relates to achieving a longer residence time inside the cells and not the endocytosis rate⁹³. The conjugation of anti-prostate specific membrane antigen (PSMA) antibodies with a generation 5 PAMAM dendrimer was shown to bind specifically and selectively to PSMA positive cells. Confocal techniques was used to image the dendrimer internalisation *in vitro*⁹⁴.

Dendrimers have also been broadly explored as DNA delivery agents for gene or antisense therapy and as an alternative to viral vectors^{77, 95, 96}. An activated dendrimer transfection reagent is available on the market, SuperFect^{TM68}. These are positively charged, amine terminated dendrimers which can interact with the phosphate groups of nucleic acids forming transfection complexes. The efficiency of these molecules is however dependent on the cell line and the presence of other transfection agents like DEAEdextran^{83, 97}.

The application of dendrimers as antibacterial and antiviral agents has been under intense development mainly due to the polyvalent nature of these molecules. The capability of sequential binding of multiple ligands on one biological entity to multiple receptors on another is known as polyvalency or multivalency (Figure 1.10). This can lead to an increase of the binding affinity due to neighbouring group effects and cooperative binding. Polyvalency can provide a tighter binding mediated by multiple interactions⁷³. Both linear and highly branched macromolecules have been studied in this context.



Figure 1.10 Mono- and polyvalent interactions (A and B respectively).

Dendrimers may exhibit a co-operative effect leading to an increase in affinity. Additionally, this polyvalency can also contribute to the increase in the specificity of a given interaction⁷⁷. The simultaneous interactions between viral haemaglutinin and the sialic acid residues of cell membranes in the attachment of influenza virus to its target cell is representative example of the increase of specificity through polyvalency^{77, 98}. The architecture of these molecules allows one group on the dendrimer surface to interact with a target. Other groups close to the first interaction then are more capable of interacting. This leads to a polyvalent interaction taking place. Multivalent binding with dendrimers was first reported with glycoclusters which bind to the asialoglycoprotein receptor. This led to a logarithmic increase on the binding affinity due to the higher number of saccharides exposed on the dendrimer⁹⁹.

Dendrimers presenting an anionic surface can compete with a microorganism to bind the cell surface⁷⁷. A dendrimer bearing mannose monomers on its surface was shown to inhibit the DC-SIGN-mediated infection on a pseudo-Ebola viral model. The DC-SIGN is a transmembrane C-type lectin expressed on dendritic cells that is capable of recognising high mannosylated proteins present in a wide range of pathogens like *Mycobacterium tuberculosis*, *Klebsiella pneumonia*, *Leishmania* amongst others¹⁰⁰. These results suggest that these dendrimers could potentially be used in treating infections¹⁰⁰.

Cationic dendrimers can potentially bind to negatively charged bacterial membranes causing cell damage and lysis⁷⁷. An example of this mode of action is the galabiosides modified dendrimers which prevent E.coli adhesion⁹⁸. However cationic polymers tend to be haemolytic to red blood cells.

As the basis for this thesis, a paper on dendrimer-glucosamine conjugates by Shaunak *et al.*, revealed their use as anti-angiogenic and immuno-modulators agents¹. It was reported that a generation 3.5 PAMAM dendrimer modified with glucosamine 6-sulfate showed anti-angiogenic effects. In the same study the modification of the same PAMAM dendrimers with glucosamine was shown to prevent the LPS-induced release of pro-inflammatory cytokines and chemokines. Furthermore, it was suggested that the

combined use of these two saccharide modified dendrimers could be used to prevent post surgical scar tissue formation¹.

As the potential biomedical applications for dendrimers has increased, the concern for the safety of their use *in vivo* increases. Many studies to assess the biocompatibility of these molecules have been reported investigating *in vitro* and *in vivo* cytotoxicity, immunogenicity, biopermeability and biostability.

In vitro cytotoxicity studies of anionic and cationic PAMAM dendrimers has generally shown that amine terminated dendrimers (i.e. cationic dendrimers) are, in general, more cytotoxic than the carboxylic acid terminated dendrimers (i.e. anionic dendrimers). Roberts et al showed that cationic PAMAMs produced a 90% cell death at a concentration of 7 ng/ml for generation 3 dendrimers. Larger dendrimers were still toxic, but less so. For example 90% cell death was observed at ~280 µg/ml for generation 5 and 100 nM (0.05147 µg/ml) for generation 7¹⁰¹. Similar results were obtained by Malik et al for a comparative study with PAMAM, DAB (poly(propylenemine), DAE (diaminoethane) and PLL (poly(L-lysine)) dendrimers¹⁰². In contrast anionic PAMAMs generations 1.5-9.5 were found to be non-toxic up to 5mg/mL.

Some of these results do show there may be some dependence of the cytotoxicity on the core, but the greater influence appears to be due to the end groups on the outer shell of these molecules¹⁰³. *In vivo* PAMAM dendrimer cytotoxicity seems to reasonably correlate with *in vitro* results⁷⁵. The surface charge seems to once again be a key feature with cationic PAMAM dendrimers presenting higher toxicity than neutral or anionic dendrimers^{75, 85, 104}. Cationic PAMAM dendrimers generation 3, 5 and 7 at maximum doses of 2.6 mg/kg, 10 mg/kg and 45 mg/kg respectively were administrated in mice either as a single dose or once a week for a total period of 10 weeks. No haemolytic activity, inflammation or granuloma formation was observed¹⁰¹ although one mouse did die after administration through intravenous injection in mice showed no toxicity up to doses of 95 mg/kg¹⁰².

Haemolytic assays with cationic PAMAMs, DAB and DAE dendrimers displayed haemolysis above 1 mg/ml concentrations. Only PAMAM dendrimers showed

generation dependency for these concentrations¹⁰² suggesting that there may be an influence to the endgroup presentation caused by interactions within these dendrimers. PAMAM and DAB dendrimers were shown to cause, even at non-haemolytic concentrations, severe changes in cell morphology after incubation for one hour^{103, 102}. In contrast, anionic DAB and generations 3.5-9.5 PAMAM dendrimers failed to show any haemolytic effect for concentrations up to 2 mg/ml, and the PAMAM dendrimers also showed no effect on cell morphology^{102, 103}.

The biodistribution and clearance of these molecules is an important and crucial aspect for the use of dendrimers parenterally. The charge of the outer shell of PAMAM dendrimers proved of importance for both biodistribution and permeability of these molecules. There were major differences observed between amine terminated and carboxylic acid terminated PAMAMs¹⁰³. Intravenous administration in mice of generations 3 and 4 PAMAM and generations 2.2, 3.5 and 5.5 PAMAM dendrimers showed that positively charged dendrimers (full generations) were rapidly cleared from circulation (less than 2% recovery after 1h) whilst anionic dendrimers (half generations) displayed longer circulation times (20-40% recovery after 1h)¹⁰³. All of the dendrimers revealed generation dependent clearance times^{102, 103}.

Tissue accumulation and distribution has also been given attention. Cationic dendrimers were shown to accumulate in different organs depending on their generation. Generation 3 PAMAM accumulated in the kidney (15% of injected dose, over 48 h) and generations 5 and 7 PAMAM in the pancreas (32% over 24 h for gen. 5 and 20% for gen. 7 over 2 h)¹⁰¹. The generation 7 PAMAM dendrimer also presented a high urinary tract excretion (46%, over 2 h and 74% over 4 h)¹⁰¹. A different study showed that 60 to 90% of the administrated dose of cationic dendrimers could be found in the liver after 1 h in comparison with anionic dendrimers where the administered dose was observed in the liver in the range of 25-70% after the same period¹⁰³.

The fact that cationic dendrimers show higher tissue association and lower transport rates than anionic dendrimers has been attributed to the possibility of the positively charged dendrimers interacting strongly with negatively charged cell membranes¹⁰⁵. It has also been shown that the increase on the generation of some dendrimer types and the

consequently on the molecular weight leads to an increase of their circulation times⁷⁷, ¹⁰⁶.

In summary, these studies show that anionic dendrimers, particularly, carboxylic acid terminated PAMAM dendrimers are potentially suitable for parenteral therapeutic applications. This was the basis for their selection in this PhD project to study glucosamine conjugated generation 3.5 PAMAM dendrimers as anti-imflammatory agents. Previous studies concerning the *in vitro* and *in vivo* toxicity of these conjugates were reported by Shaunak et al. and in a previous PhD thesis from the same group^{1, 107}. Effort is now directed towards attempting to gain a better insight into the molecular basis of the biological activity of the glucosamine conjugated PAMAM dendrimers. A computational approach was used to achieve this goal.

Molecular Modelling Methods

Molecular modelling is a branch of computational chemistry that studies molecules by applying fundamental laws of physics and chemistry¹⁰⁸. It aims to predict the behaviour of individual molecules within a chemical system as represented by the Schrödinger equation¹⁰⁹,

$$i\hbar \frac{\partial}{\partial t}\Psi = \hat{H}\Psi$$

where Ψ represents the wave function, $i\hbar \frac{\partial}{\partial t}$ the energy operator and \hat{H} the Hamiltonian.

This equation has a time dependent form and a time independent form. The time dependent form refers to the motion in a quantum state and for one dimension is represented as,

$$i\hbar \frac{\partial}{\partial t}\Psi(x,t) = \hat{H}\Psi(x,t)$$

The time independent equation refers to standing waves, in definite quantum states, where E is the energy of the particle,

$$E\Psi = \hat{H}\Psi$$

Since the Schrödinger equation for systems larger than the hydrogen atom can be solved only numerically, it is essential to introduce approximations. The size and the complexity of molecular systems that are studied require the use of approximations. These approximations have come to categorise molecular modelling techniques into three general types: *ab initio*, semi-empirical and empirical methods¹⁰⁹.

Ab initio methods

Ab initio molecular modelling methods use the highest level of theory to solve the Schrödinger equation for a given molecule. It uses exclusively mathematical based approaches with appropriate approximations¹¹⁰. However, this purely mathematical approach is limited by the computational power available. A molecular system of about 100 atoms is considered the maximum number of atoms for accurate calculations in 2007^{110} , though these values are constantly changing as more powerful computers and more efficient algorithms are developed.

The two main concepts involved in using these methods are the level of theory and the basis set. The choice of the model is a determinant step for the use of these methods where a compromise between the level of theory, the extent of the basis set and the size of the system needs to be done as it will greatly influence the computational time.

The most common method, corresponding to the level of theory, is the Hartree-Fock method. Although the theory was developed in the 1920's this approach has been more widely used since the 1950's when electronic computers begun to be more widely used and develop.

The Hartree-fock theory is a basic theory that is only applicable near equilibrium states. It is particularly useful when examining relatively large organic molecules. Its mathematical derivatisation is beyond the scope of this work but the molecular wave function defined by Schrödinger's equation is, as above,

$$\hat{H}_{mol} \Psi_{mol} = i\hbar \frac{\partial \Psi_{mol}(t)}{\partial t}$$

 \hat{H} represents the Hamiltonian operator, Ψ the molecular wave function and \hbar the reduced Plank's constant. The Hartree-Fock theory constructs the wave function by assigning pairs of electrons with opposite spins to molecular orbitals, ϕ (r) and using a determinant

with two spin functions α and β , which is also known as the Slater determinant. For n electrons in n/2 orbitals,

$$\Psi_{mol} = \frac{1}{\sqrt{n!}} \begin{vmatrix} \phi_1(1)\alpha(1) & \phi_1(1)\beta(1) \cdots & \phi_{n/2}(1)\alpha(1) & \phi_{n/2}(1)\beta(1) \\ \phi_1(2)\alpha(2) & \phi_1(2)\beta(2) \cdots & \phi_{n/2}(2)\alpha(2) & \phi_{n/2}(2)\beta(2) \\ \vdots & \vdots & \vdots \\ \phi_1(n)\alpha(n) & \phi_1(n)\beta(n) \cdots & \phi_{n/2}(n)\alpha(n) & \phi_{n/2}(n)\beta(n) \end{vmatrix}$$

This theory is based on the principle that each electron moves in an average potential of the remaining electrons without knowledge of their positioning^{70, 111}. Thus electron motion due to Coulombic interactions is neglected.

The main drawback of this theory is the lack of electron correlation and the overestimation of repulsion. This often produces a certain level of inaccuracy in the calculations¹¹¹.

There are however different theories which are generally known as post-Hartree-Fock theories that include electron correlation. An example is the Møller-Plesset perturbation theory, which assumes electron correlation as a minor effect and introduces small perturbations to the Fock operator¹⁰⁹. Alternatively, the density functional theory (DFT) represents electron correlation as a function of electron density. In fact, the most common approaches depend on the use of hybrid methods between HF and DFT, such as B3LYP¹¹² and PW9¹¹³.

The second important step in conducting *ab initio* calculations is the choice of the basis set. Basis sets are contracted Gaussian functions which represent molecular orbitals¹⁰⁹. They can be classified into different categories that included minimal, split valence, polarised, diffused and high angular momentum basis sets.

Minimal basis set are the simplest and contain only a minimal number of functions that are needed to characterise an atom, for example, one function for hydrogen and 5 functions for lithium. The most common basis set of this type is STO-3G which represents Slater-Type Orbitals by a combination of 3 Gaussian functions.

Split valence basis sets take into account valence electrons as the ones that are involved in chemical interactions. This type of basis set uses a simplistic approximation for the behavior of shielding or core electrons and a more detailed approach to valence electrons^{114, 115}.

Polarised basis sets add polarisation functions to valence electron shells to allow for charge displacements from the nuclei. The $6-31G^*$ is a polarised basis set and it was the one chosen in this PhD project. This set takes into account an inner shell atomic orbital with a contracted Gaussian function made of six primitive Gaussian functions, and an inner valence shell with a contracted Gaussian function made of of one primitive Gaussian functions. The outer valence shell is comprised of one primitive Gaussian function and a single set of Gaussian polarization functions^{115, 116}.

Diffusion basis sets add diffusion functions to valence electron shells that allow for a more accurate treatment of weakly bound electrons. Finally, higher angular momentum basis sets apply more than one polarisation function in addition to the valence electron shells. These basis sets are generally used with post-Hartree-Fock methods.

Semi-empirical methods

Semi-empirical methods use approximations of *ab initio* techniques that have been fitted to experimental data in order to reduce the computational resource. These methods use extensive approximations and require *ab initio* or experimental data as parameters. They allow the analysis of more complex molecular systems with hundreds of atoms¹⁰⁹. They were primarily developed to predict the properties of organic molecules¹¹⁷.

Semi-empirical methods are usually classified according to their treatment of electron-electron interactions. One of the most well known early methods is the Extended Hückel Method, which neglects all electron-electron interactions. It is suitable for the determination of molecular orbitals and their relative energies but does not allow for structural geometry determination¹¹⁸.

Neglect of differential overlap models (NDO) use different approximations to solve Schrödinger's equation with the Hartree-Fock method¹⁰⁹. The simplest is the complete NDO (CNDO) which neglects almost all electron exchange properties. Thus CNDO does not differentiate between states in the same electronic configuration except for spin values¹⁰⁹. The intermediate NDO (INDO) and the modified INDO (MINDO) neglect differential overlap between orbitals on different atoms. These methods have shown reasonable results with organic molecules¹¹⁹ to aid in predicting enthalpies of formation and geometries. Finally, Zerner's INDO methods or ZINDO, comprises a reparameterisation of INDO specifically to reproduce electronic spectra results. This method is useful to predict UV spectra but not suitable for geometry optimization¹¹⁹.

A different set of methods is based on the neglect of diatomic differential overlap (NDDO). Its origin is based on the INDO model but includes interaction between overlap densities on the same or two different atoms¹⁰⁹. There are three representative models of this kind, MNDO, AM1 and PM3. The Modified NDO¹²⁰ (MNDO), was implemented to overcome some limitations of MINDO and though it shows good results with some organic systems it underestimates excitation energies and overestimates barriers to chemical reeactions^{109, 119}.

The Austin methods version 1¹²¹ (AM1) is available in almost all semiempirical software and is one of the most accurate methods along with the parameterisation model 3 (PM3)^{119, 122}. However, AM1 is not able to deal with nitro compounds, peroxide bonds and phosphorus-oxygen bonds¹⁰⁹. Finally, PM3¹²³, is very similar to AM1 but with improved parameterisation and it is capable of dealing with hydrogen bonds in a more efficient manner than AM1. PM3 is able to estimate more accurately the heats of formation, energies and bond lengths¹²³. There are limitations of PM3 for example, non-bonded distances are sometimes too short and polycyclic rings are not always flat¹²⁴. More recently, a new parameterisation, PM6, has been developed¹²⁴. This development has been incorporated into MOPAC¹²⁴ which is frequently used for semi empirical calculations. PM6 contains new *ab initio* data for main group elements and transition metals and it shows improvements compared to PM3 and AM1 in relation to hydrogen bond treatment, fused ring planarity and improvements in gas phase energy predictions¹²⁴.

Molecular mechanics methods

These two first molecular modelling categories concern quantum mechanics approaches and the last category is an empirical or molecular mechanics method. Molecular mechanics methods are based on the classical laws of physics to predict chemical properties. They neglect explicit treatment of electrons and are not able to deal with problems where electronic and quantum effects prevail such as bond formation or cleavage¹⁰⁹. The potential energy of the system under study is determined using force fields¹²⁵. Molecular mechanics techniques treat atoms within molecules as variable masses connected by springs and states that the coordinates of these atoms are possible to determine in the place where the springs are in equilibrium¹⁰⁹. In reality, these atoms are subject to a combination of repulsion and attraction forces and these contributions form the basis of the force fields¹²⁵. This is described in more detail in Chapter III. These methods allow the study of much larger systems with thousands of atoms and can be used for geometry optimisation and minimisation, and non-electronic properties prediction.

Molecular dynamics and biosimulation

Molecular dynamics methods calculate the forces between the atoms at each time-step and from that these methods are designed to calculate the new positions and velocities of all the atoms, by applying Newton's laws of motion¹²⁶.

$$\vec{F}_{\iota} = m. \vec{a}_{\iota}$$

Where $\vec{F_i}$ represents the applied force, *m* the mass and $\vec{a_i}$ the acceleration.

The position of atoms in time increments are based on coordinates of each atom and calculated using molecular mechanics methods and forces at each time-step, allowing the generation of a motion (molecular simulation) trajectory¹²⁶.

$$\frac{d^2 x_i}{dt^2} = \frac{F x_i}{m_i}$$

The equation represents the motion of a particle m_i along a coordinate x_i with an applied force F^{126} .

The assumption of these methods is that all atoms can be treated as classical particles within the Born-Oppenheimer approximation¹²⁷. This assumes separation of nuclear and electronic motions¹²⁸. Under this approximation it is assumed that if the forces applied to both nucleus and electrons are from the same order of magnitude than, under nuclear

motion, electrons move at such velocity that will rapidly relax to the ground state¹²⁸. The nucleus is much heavier than the electrons and thus should move with significantly lower velocity¹²⁸. Therefore it is plausible to assume the nucleus is stationary and solve the time independent Schrödinger's equation for electrons in the ground state first followed by determination of the energy of the system, in that configuration, for the motion of the nucleus^{128, 129}.

Molecular dynamics methods can be applied to large biomolecular systems, however, the study of large systems is computationally very demanding. To overcome computational resource constraints molecular dynamics methods utilise approximations that allow quicker calculations without major consequences on the determination of the potential energy of the system¹²⁶. The most commonly used approximations are periodic boundary conditions, non-bonded cutoffs and Particle mesh Ewald.

Periodic boundary conditions are an important parameter for biosimulation, where solvation needs to be considered to better reproduce *in vivo* conditions. In this approximation the target molecules and the solvent molecules are placed in cell. The solvent molecules are represented using different models. The most commonly used are TIP3 and SPC¹³⁰. The cell with the fully solvated system is assumed to be replicated infinitely by a rigid translation in all Cartesian directions¹³¹. This cell can be cubic, hexagonal, octahedral, rhombic dodecahedral or elongated dodecahedral, to allow tessellation (division of a geometrical object into smaller polygons with no gaps or overlaps) in 3-dimensions. This enables the particles in a cell to experience forces as if in a much larger bulk fluid. When an atom moves out of the cell it is replaced by an image particle that enters from the opposite side¹²⁶.

Non-bonded terms are the most demanding contribution in terms of computational time. In a force field the number of bonded terms is proportional to the number of atoms in a molecule, however, non-bonded terms is of an order of N^2 . The Lennard-Jones potential describes the interaction between two atoms:

$$V(r) = 4\epsilon \left[\left(\frac{\sigma}{r}\right)^{12} - \left(\frac{\sigma}{r}\right)^{6} \right]$$

where ϵ represents the depth of the potential well, σ is the distance where the interparticle potential is zero and r the distance between particles. The r^{-12} term represents the Pauli repulsion principle due to overlapping electron orbitals and the r^{-6} term describes the van der Waals forces¹³¹. However this potential decreases very rapidly with distance, so the most computationally effective way of dealing with these terms is by implementing a *non-bonded cutoff*. The generally recommended value is 10 Å¹²⁶.

Electrostatic interactions cannot be treated in the same manner, as these decrease inversely proportional with distance $(r^{-1})^{126}$. The most commonly used approximation to represent electrostatic interactions is implemented in the Particle Mesh Ewald (PME) method. This method is based on a theoretical chemistry method known as Ewald Summation¹³², where each particle in the system interacts with all other particles within the box and their images in the periodic cells¹²⁶. The potential is distributed in two terms; a short ranged space or real space and the longer range or Fourier space:

$$\varphi(r) = \varphi_{sr}(r) + \varphi_{lr}(r)$$

The PME method allows for the replacement of the direct sum of the interaction energies (Eq.1) with the two terms. The first term accounts for the particle (short range interactions – Eq 2) and the second term accounts for the Fourier space (long range – Eq.3):

$$E_{total} = \sum_{i,j} \varphi(r_j - r_i) = E_{sr} + E_{lr} \qquad \text{Eq. 1}$$

$$E_{sr} = \sum_{i,j} \varphi_{sr} (r_j - r_i)$$
 Eq. 2

$$E_{lr} = \sum_{k} \Phi_{lr}(k) |\tilde{\rho}(k)|^2 \qquad \text{Eq. 3}$$

The Fourier transforms are represented by $\Phi_{lr} \tilde{\rho}(k)^{132, 133}$.

Both particle and the Fourier space terms converge, allowing their truncation and leading to significant improvement on the computational time without great loss of accuracy^{126,132,133}.

Once it is possible to determine the energy values for each time step, there is a need to generate the trajectory. The finite difference method integrates the equations of motion and determines the future position¹²⁶. In this method integration is broken into small stages separated by length δt and the motion equation integrated between t and t + δt with constant force. The forces in the new positions are determined leading to new positions at time and velocities $t + 2\delta t$, et cetera.

There are many algorithms to integrate the motion equations. The frequently used is the Verlet algorithm. However, all algorithms assume that positions, velocities and accelerations can be approximated as a Taylor series:

$$r(t + \delta t) = r(t) + \delta t v(t) + \frac{1}{2} \delta t^2 a(t) + \frac{1}{6} \delta t^3 b(t) + \frac{1}{24} \delta t^4 c(t) + \dots$$
$$v(t + \delta t) = v(t) + \delta t a(t) + \frac{1}{2} \delta t^2 b(t) + \frac{1}{6} \delta t^3 c(t) + \dots$$
$$a(t + \delta t) = a(t) + \delta t b(t) + \frac{1}{2} \delta t^2 c(t) + \dots$$
$$b(t + \delta t) = b(t) + \delta t c(t) + \dots$$

where \mathbf{v} is the vector velocity (first derivative of the positions with respect to time t), \mathbf{a} the acceleration (second derivative) and \mathbf{b} is the third derivative, *etcetera*.

In biomolecular simulations the solvent is not always relevant, especially in regions far from the solute molecule. In these cases methods that use implicit solvation can be applied. This can decrease the computational time required for useful solutions. Stochastic dynamics methods are frequently used for this purpose. They assume the force on a particle has three components: (i) interaction of the particle with other particles, (ii) motion of the particle through the solvent and (iii) random fluctuations due to interactions with solvent¹²⁶. This method is usually used in conjunction with Generalized Born Surface Area (GB/SA) continuum model for solvation, with a solvation energy expressed as a sum of pair-wise interactions among intramolecular atoms¹³⁴.

Aims of the Project

It is hypothesised that computational techniques can be used to gain a greater insight about the structure property correlations of the glucosamine conjugated PAMAM dendrimers that have been shown to inhibit the LPS mediated pathways. This project aims specifically to apply molecular modelling techniques to the structural study of glycosylated generation 3.5 PAMAM dendrimers and their interaction with the TLR4-MD-2 system. This effort is focused on determining whether better and more active dendrimers with preserved biological activity can be developed. For this purpose a series of steps were taken with different objectives:

- Expand the knowledge of the PAMAM dendrimer system, through the use of molecular modelling techniques, to characterise the mixture of conjugates that resulted from the synthesis of the glucosamine modified dendrimers and synthesis of a different conjugate with a saccharide that is more similar to the saccharide on Lipid A.

- Understand the loading and distribution of the glucosamine molecules on the generation 3.5 PAMAM dendrimer as well as their dynamic behaviour using semiempirical and molecular dynamic studies.

- Understand the mechanism of action of these glycosylated dendrimers and their interaction with the TLR4-MD-2 complex through the use of docking studies.

- Develop parameters that allow the distinction between active and non active dendrimeric structures and rationally design better molecules.

CHAPTER II – SYNTHESIS, CHARACTERISATION AND BIOLOGICAL EVALUATION OF SACCHARIDE MODIFIED PAMAM DENDRIMERS GENERATION 3.5

Introduction

In 2004, Shaunak and co-workers reported that dendrimer glucosamine conjugates exhibited immuno-modulatory activity and can prevent the progression of LPS triggered inflammatory responses¹. The synthesis of the glycosylated dendrimers simply employed a routine carbodiimide mediated coupling of an amino-saccharide (i.e. glucosamine) with commercially available carboxylated terminated PAMAM dendrimers. The carboxylic acid terminal groups of the dendrimer were activated by the coupling agent EDC and the activated PAMAM dendrimer then underwent nucleophilic attack by the amine of the saccharide.



Figure 2.1 Synthesis of dendrimer glucosamine. 1 - Carboxylic acid activation by EDC. 2 - Nucleophilic attack by the amine group of the glucosamine occurs at a carbodiimide derived activated ester moiety on the dendrimer resulting in the formation of the glycosylated dendrimer. The urea of EDC is formed as a co-product.

The dendrimer glucosamine conjugates were synthesised using a divergent strategy (Figure 2.2). In this way, the dendrimer is build from the core to the surface and then modified with the saccharide.



Figure 2.2 Generation 3.5 PAMAM glucosamine conjugate.

In this particular case the generation 3.5 PAMAM dendrimers with a carboxylated surface were purchased and the saccharide conjugation was performed as described above (Figure 2.1). The divergent synthetic route has some disadvantages. It does not allow any control of the regiochemistry of the saccharide conjugation on the dendrimer surface. In principal, each of the dendrimers will have the glucosamine conjugated at different carboxylates on the surface. The number of glucosamine molecules per dendrimer, i.e., the glucosamine loading also varies. The result is a mixture of dendrimer conjugates that will not be amenable to precise characterisation either by NMR or mass spectrometry. There will be a superposition of peaks and ionisation profiles respectively. A convergent synthetic strategy starts from the dendrimer end groups with chemical reactions ending with bonds to the core being formed. This allows more control of surface regiochemistry and in the case of glucosamine conjugation, control in the

number of glucosamine molecules per dendrimer (i.e. loading). The possibility of conducting a convergent synthesis¹³⁵ was considered for this project, however, this would be a much more time and cost demanding approach and there were no guarantees that the final product would maintain the desired biological activity. The previously published results showed that the glucosamine dendrimers prepared by the divergent approach did display a unique biological profile¹. Those results also showed that the synthetic conditions used did not provide a means to completely glycosylate the dendrimers, so it was hypothesised that some control due to steric interactions on the dendrimer surface may have some influence on the reaction outcome.

The approach in this project was also driven by the need to perform the conjugation of the saccharides on to the dendrimers in water. Use of an organic solvent needed for convergent synthesis could potentially lead to difficulties in complete removal of solvents and thus affecting the biological evaluation. This is particularly important in case the final product is to be considered as a medicine. There was also an additional concern that some of the organic solvents that would be used were not available as endotoxin free preparations. A tedious purification process, to remove trace organic solvent that at the same time might contaminate samples with endotoxin, was not wanted. The presence of exogenous endotoxin during the biological evaluation of the glucosamine dendrimer conjugates would mask their activity. To minimise endotoxin contamination and the need to remove large amounts of endotoxin prior to biological evaluation, the coupling reaction was conducted in endotoxin-free water that is normally used for injection into patients. Similarly all dialysis steps used endotoxin-free water that is normally used for patient dialysis (Baxter Healthcare).

There are some disadvantages to this approach. The by-product formation to give the EDC derived urea occurs readily in water. Therefore, larger stoichiometry of EDC in the reaction is necessary but it also results in an increase in the formation of the urea. Removal of the urea during purification can therefore be an issue. A side-product from the coupling reaction is an acylisourea adduct that acts as a terminating product. Since multiple reactions occur on each PAMAM dendrimer the potential for a broad distribution of products exists. To minimise formation of the acylisourea, pH of the EDC coupling reaction was lowered to 5. This is often cited as an optimal pH for this

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reaction because the activated ester is protonated on the carbodiimide derived Sp2 that is thought responsible for acylisourea formation¹³⁶.

The characterisation and purification of the dendrimer glucosamine conjugates was first conducted as described in the previously published protocol¹ and the sample was analysed by NMR and MALDI-TOF. Since the presence of impurities had been detected (e.g. EDC urea), all the reagents involved in the synthesis were analysed by the same techniques. A new purification technique was then developed for new batches of dendrimer glucosamine to be synthesised. Finally, the synthesis of a new dendrimer conjugate derived from a different saccharide, N-acetylglucosamine, was performed, Again there were some changes required compared to previously published protocol. These new dendrimer conjugates were then evaluated for biological activity.

Therefore the aim of the experiments described in this chapter was to investigate and to further develop methods for the synthesis and characterisation of the saccharide modified dendrimers. Additionally the synthesis of a new generation 3.5 PAMAM dendrimer modified with an N-acetylglucosamine derivative was undertaken with the aim that this new conjugate would also provide more of an insight about how these saccharide modified dendrimers might inhibit cytokine and chemokine release. Careful characterisation of the dendrimer conjugates would provide experimental data to inform molecular modelling work with the intent to acquire a greater understanding of the biologically important structural features of these molecules (eg molecular shape in solution, saccharide loading, charge distribution, hydrophilicity, etc). Those essential features could be incorporated during computational analysis to aid in the rational design of new molecules with desired biological activity.

Experimental Section

Starburst PAMAM derived from a diaminobutane core with a sodium carboxylate surface, Gen 3.5 dendrimers were purchased from Dendritic Nanotechnologies INC, USA. The glucosamine hydrochloride was obtained from Sigma-Aldrich, UK, the endotoxin-free water from Baxter Healthcare Limited, UK and the cross linking agent EDC was obtained from Lancaster synthesis, UK. For the purification of the reaction mixture both chromatography and dialysis techniques were used. The Slide-A-Lyzer Dialysis Kit was purchased from Thermo Scientific, USA and the Sephadex G25 from Sigma-Aldrich, UK. The endotoxin removal columns contained detoxi-Gel polymixin B obtained from Pierce, USA and were regenerated with a 1% sodium deoxycholate solution obtained from Sigma-Aldrich, USA. The filters used were 0.2 µm filters obtained from Milipore, USA. For the MALDI analysis the matrix 2,5dihydroxybenzoic acid was obtained from Sigma-Aldrich, USA, the acetonitrile was obtained from Rathburn, UK and the water was filtered through a Milli'Q'grade filter from Millipore, USA. The calibration mixture was obtained from Applied Biosystems, USA. The 2-acetamido-2-deoxy- β -D-glucopyranosyl amine for the new conjugate synthesis was obtained from Carbosynth, UK. For the biological evaluation assays the RPMI 1640 media and streptomycin were obtained from Invitrogen, UK. The Ficoll-Histopaque for PBMNs isolation was obtained from GE Healthcare, UK. For the haemolysis assay, Amphotericin B was obtained as Fungizone from Bristol-Myers Squibb, USA. The RNA was extracted using a RNeasy Mini Kit from Quiagen, UK and the reverse transcription reaction performed with a Quantitech® Reverse Transcription kit obtained from Quiagen, UK. For the real time PCR experiments the Sybr-Green JumpStart real-time PCR mix used was obtained from Sigma-Aldrich, USA and the PCR microtubes from Corbett / Qiagen, UK.

Where possible the entire synthesis was performed using sterile endotoxin-free material, disposable pipettes and containers and all glassware and magnetic stirrers were autoclaved twice to destroy LPS.

Synthesis of dendrimer glucosamine

The Starburst PAMAM derived from a diaminobutane core with a sodium carboxylate surface, Gen 3.5 dendrimers were supplied in a 10% w/w solution in methanol and 1.52 ml of this solution was aliquoted and the methanol evaporated under a nitrogen stream. The solid dendrimer was then dissolved in 1.52 ml of sterile endotoxin-free water to a concentration of 100 mg/ml. Glucosamine hydrochloride (66.8 mg) was dissolved in sterile endotoxin-free water (1.3 mL). Dendrimer at 1.2 mM and glucosamine at 0.5 mM (26.4 molar equivalents) were mixed together under continuous stirring at room temperature and pH 5 for 2 h in the presence of 5 mM 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (278.3 mg in 5.6 mL, 4.7 molar equivalents). The pH was verified every half an hour with a pH meter and, when necessary, adjusted using a 1N HCl solution. The resulting 8.42 ml of reaction mixture was purified using three different strategies.

Purification of dendrimer glucosamine

Removal of the unreacted reagents (EDC) and reaction by-products was accomplished by dialysis and chromatography. The purity of the dendrimer products was determined by NMR and MS analysis.

Purification of dendrimer glucosamine with dialysis

The dendrimer glucosamine reaction mixture (8.42 mL) was injected into a dialysis cassette and this was introduced into a beaker with 2.0 L of endotoxin-free patient dialysis H_2O and kept at 4°C with continuous stirring. Aliquots (1.0 mL) were collected after 1, 17, 41 and 65 hours for NMR, MS and biological assays. The water was changed after each aliquot was removed.

Purification of dendrimer glucosamine with chromatography

Sephadex G25 powder was rehydrated overnight in excess sterile water and a bed volume of 10 ml of Sephadex G25 was loaded into 20 syringes plugged with glass wool. The columns were then spun in a centrifuge at 1500 rpm (400 g) for 2.0 min and the volume of flow through measured - giving an approximate void volume of 4.0 ml. The 8.42 ml of the dendrimer glucosamine conjugates were separated into two fractions

loaded down two separate columns and the columns centrifuged for 2 min at 1000 rpm, fractions were collected in 50.0 ml sterile centrifuge tubes, the procedure was performed twice.

Purification of dendrimer glucosamine using a combination of chromatography and dialysis

An aliquot of 1.0 ml of dendrimer glucosamine conjugate (not purified) was injected into a dialysis cassette and this was introduced to a glass flask with 2.0 L of dH₂O and kept at 4°C with continuous stirring for 48 h. The water was changed every 24 h. The remaining 7.42 ml were eluted through Sephadex G25 columns, as described above. An aliquot of 3.0 ml of Sephadex purified dendrimer glucosamine conjugate (purified) was injected into a dialysis cassette and this introduced into a glass flask with 2.0 L of dH₂O and kept at 4°C with continuous stirring for 48 h. The water was changed every 24 h. All fractions, regardless of the purification method were then eluted through endotoxin removal columns¹³⁷.

Endotoxin removal columns

All fractions of the purified conjugate were passed down two detoxi-Gel polymixin B containing columns to remove endotoxin. The columns were regenerated with 5.0 ml of a 1% sodium deoxycholate solution and eluted with 5.0 ml endotoxin free water. The final product was filter sterilised with a 0.2 μ m filter and freeze-dried at -80°C. Dendrimer glucosamine powder was dissolved to 50 mg/ml and stored at 4°C.

NMR analysis

The NMR analysis of dendrimer glucosamine gen. 3.5 and all the reagents involved in its synthesis was performed using a Bruker AVANCE 500MHz NMR spectrometer. The samples were diluted in 90% $H_2O/10\%$ D₂O solution and TMS was used as an internal standard. The analysis was performed at 323 K. All samples were submitted for ¹H-NMR, ¹³C NMR, DEPT, COSY, TOCSY, NOESY, HMQC and HMBC. For the analysis of the dialysed dendrimer conjugates, ¹H-NMR was performed using a Bruker AVANCE 400 MHz spectrometer, using the same solvent ratio, TSP standard and

temperature as described above. The number of transients used was 256 for all experiments.

MALDI-TOF analysis

The mass spectra of the dendrimer glucosamine gen 3.5 and all the reagents involved in its synthesis were acquired using a PE Biosystems Voyager-DE STR MALDI-TOF mass spectrometer using a nitrogen laser operating at 337.0 nm. The matrix solution was freshly prepared 2,5-dihydroxybenzoic acid at a concentration of 10 mg/ml in a 50:50 mixture of acetonitrile and water. This was added to the sample to a range of serial dilutions between 0.2 and 0.02 nmol of sample. Sample and matrix, 0.5 ul of each, were spotted onto the sample plate. The spectrometer was calibrated using the calibration mixture run as a close external standard. At least 100 shots per spectrum were acquired over the range 5,000 - 20,000 Da for the dendrimer conjugate and 2,000 – 20,000 Da for the reagents, under linear conditions with an accelerating voltage of 25,000 V, and extraction delay time of 750 nanoseconds. Data processing was performed using Voyager-DE Pro Data Explore software.

Synthesis of the gen. 3.5 dendrimer conjugates derived from 2-acetamido-2-deoxy-

β-D-glucopyranosyl amine

Starburst PAMAM with a diaminobutane core and sodium carboxylate surface, Gen 3.5 dendrimers were supplied in a 10% w/w solution in methanol. From this solution 1.0 mL was aliquoted and the methanol evaporated under a nitrogen stream. Dendrimer was then allowed to dissolve in 1.0 mL of sterile endotoxin free water. The 2-acetamido-2-deoxy- β -D-glucopyranosyl amine (40.7 mg) was dissolved in sterile endotoxin free water (814uL). To the sterile aqueous solution of the dendrimer (1.0 mL, 1.2 mM) was added a sterile aqueous solution of glucosamine (814uL, 0.5 mM, 26.4 equivalents). The pH of this solution was maintained at 5.0, adjusting with a 1N HCl solution whenever necessary, and was stirred continuously while a sterile aqueous solution of EDC (5.6 mL, 5 mM, 4.67 equivalents) was added intermittently over a 3.0 h period. The final volume of 7.4 ml of the dendrimer conjugate was purified with 48 h dialysis as

described above. After endotoxin removal they were submitted to biological evaluation as described for dendrimer glucosamine.

Biological assays

Media

The culture medium was RPMI 1640 containing 330 μ g/ml L-glutamine, 200 IU/ml penicillin and 200 μ g/ml streptomycin supplemented with 10% heat inactivated human serum.

Source and isolation of human leukocytes

Leukocytes were isolated from overnight peritoneal dialysis bag effluents. The source of macrophages was from pooled buffy coat residue blood from donors (National Blood Transfusion Service Collindale). PBMNs were isolated by layering over Ficoll-Histopaque and centrifugation for 30 min at 2000 rpm (800 g). The macrophages were isolated by adhesion to 14 cm tissue culture plates at 37° C, 5% CO₂ in culture media for 1.0 h. All non-adherent cells were removed by 2 x 20.0 mL washes with PBS (phosphate buffered saline) and macrophages harvested by scraping with a cell scraper in 10.0 mL of PBS and centrifuged (350 g, 5 min). Cell pellets were resuspended in media and counted using a Coulter counter.

Cytotoxicity assays - MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to determine cytotoxicity of the dendrimer glucosamine conjugates. Dendrimer glucosamine at a concentration 800 μ g/ml was added to triplicate wells in the 96-well flat-bottomed plates (Greiner Bio-one, UK) in media and serially diluted 2 fold in 100.0 ul media to give a final concentration of 0.2 μ g/ml. Control wells did not contain dendrimer conjugates (100% viability). Serial dilutions of Triton 1% were added to the cells in triplicate wells as an external control to give 100% cell killing. Cells (100,000 cells/well in 100.0 μ l culture media) were then added. Plates were incubated overnight at 37°C, 5% CO₂. 20 μ l/well of MTT reagent (5.0 mg/mL) in PBS was added with 3.0 h

of incubation with the same conditions. Plates were centrifuged at 450 g for 5 min, the supernatant was removed and 100 μ l DMSO was added to dissolve the dark blue precipitate that was generated. The optical density was then measured at 570 nm in a spectroscopic plate reader (u-Quant, Bio-Tek Instruments) and the percentage of cell viability relative to the control cells determined.

Haemolysis assay

This assay was performed to assess the toxicity of the dendrimer conjugates to red blood cells. Duplicate serial dilutions of the dendrimer glucosamine were performed to a range 800-3.13 µg/ml with media without HS (human serum) in a final volume of 100 µl/well in a 96-well round bottom plate. Quadruplicate wells of cells without dendrimer glucosamine were used as negative controls. The positive control was serial 1:2 dilutions Amphotericin B to a concentration range between 800-0.8 µg/ml, performed in duplicate. Four wells containing only media were used as blanks with another four containing Triton X-100 1% as a 100% haemolysis reference standard. One hundred µl of a 2% w/v solution of red blood cells was added to each well except for the blank. The plate was incubated for 1h at 37°C, 5% CO2. After incubation, the plate was spun down at 850 g for 10 min and 100 µl extracted from each well placed into a new plate and the OD read at 490 nm.

Assays for anti-inflammatory activity of dendrimer construct in an LPS – monocyte model system

Incubation with LPS and dendrimer glucosamine conjugates

Freshly isolated PBMNs or monocytes in 1000 μ l culture medium were seeded into 48well culture plates in duplicate at 5 x 10⁵ cells/well. The PBMNs or monocytes were used immediately or cultured at 37°C, 5% CO₂ for up to 24 h. Dendrimer glucosamine at a final concentration of 25-200 μ g/ml was then added for 1 h at 37°C, 5% CO₂. LPS at a final concentration of 25.0 ng/ml was then added to the cells which were incubated for a further 3 h. Positive control wells were stimulated with LPS in the absence of dendrimer glucosamine whilst negative control wells were cultured with dendrimer glucosamine in the absence of stimulation with LPS.

RNA extraction and reverse transcription

The RNA extraction was performed with RNeasy Mini Kit extraction kit according to the manufactures' instructions. The RNA concentration was measured on a UV 1101 Biotech Photometer (Jensons-Pls, UK). RNA was stored at -20°C. The extracted mRNA was converted to cDNA using a Quantitech® Reverse Transcription kit according to the manufactures' instructions. The product of the reaction was diluted 1:4 and stored at -20°C for further experiments.

Real time PCR (RT-PCR) quantification of chemokine and cytokine cDNA

PCR amplification was performed in a Corbett R3000 Rotor-Gene machine. For the purposes of quantification, plasmid standards over the range $20 - 2 \times 10^7$ copies per PCR reaction were simultaneously amplified with the target cDNA. Each real-time PCR reaction mix contained 1 µl (final concentration 0.25 µM) of each of the desired forward and reverse primers, 6.0 µl water and 10.0 µl of 2X Sybr-Green JumpStart real-time PCR mix. The reaction mixes were added to PCR microtubes and 2 µl diluted cDNA (or plasmid standard) added for amplification. The steps in the RT-PCR cycle are described in Table 2.1.

Temperature (°C)	Duration (s)		
95	180 denaturation (one cy	180 denaturation (one cycle,)	
94	5 (denaturation)	40 cycles	
59	2 (annealing)		
72	8 (extension)		
81-84	5 (acquisition)		
95	5		
65	10		
65-95 melting curve analysis	0.1°C per s (one cycle constant acquisition)		

Table 2.1 Real time RT-PCR sto	eps in the Corbett RG-3000 rotor ge	ene.
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Chemokine / Cytokine	Forward primer 5'-3'	Reverse Primer 5'-3'	Annealing temperature (°C)	Acquisition temperature (°C)
MIP-1a	TGCTGCTTCAGCTACACCTC	GCACTCGGCTCCAGGTCACT	59	84
MIP-1 β	ACCCTCCCACCGCCTGCTGC	GTTCCAGGTCATACACGTACTCC	59	84 - 85
IL-8	CCTTTCCACCCCAAATTTATCA	GCCCTCTTCAAAACTTCTCCA	59	81
TNF- α	AGGCGGTGCTTGTTCCTCA	GTTCGAGAAGATGATCTGACTGCC	59	83
IL-1 β	CCCACAGACCTTCCAGGAGA	CGGAGCGTGCAGTTCAGTG	59	81 - 84
IL-6	CACACAGACAGCCACTCACCTC	CTGCCAGTGCCTCTTTGCTG	59	81 - 82

 Table 2.2 Chemokine and cytokine primers, annealing and acquisition temperatures.

Results and Discussion

The current study started with the synthesis and characterisation of the previously reported dendrimer glucosamine conjugates¹. The key of objective of this PhD project was the computational analysis of the dendrimer glucosamine conjugates to try to gain some insight into their biological mode of action. However it was important to prepare and evaluate the dendrimer conjugates again to ensure that it was possible to reproduce the biological activity that have been reported previously. Also the dendrimer used in these studies had a different core to the one in the previously published report. The new dendrimers had a diaminobutane core, which Dendritic Nanotechnologies Inc, claimed had higher purity in relation to the EDA core which was previously used¹³⁸. Modifications in the core were not important since the surface modifications confer the biological activity.

Characterisation of the product of dendrimer glucosamine gen 3.5 synthesis

The ¹H-NMR analysis of the dendrimer glucosamine obtained by the initial EDC mediated conjugation reactions revealed the presence of impurities (Figure 2.3). These could originate from the starting materials or from side reactions. For example, the generation 3.5 PAMAM dendrimer could contain impurities even though it was purchased as a monodispersed product and information from the supplier was not indicating presence of impurities. Furthermore, the synthetic strategy used for saccharide conjugation does not allow for any control of the level of conjugation. The use of EDC in water also may result in an increase in side reactions with N-acyl isourea formation.



Figure 2.3. ¹H 1D NMR spectrum of a gen. 3.5 dendrimer glucosamine. a) C-1 H_{α} of glucosamine; b) overlap of peaks belonging to the CH₂ groups of the glucosamine and of the dendrimer; c) and d) EDC peaks.

The spectrum obtained for the gen. 3.5 dendrimer glucosamine was compared to those of the reagents involved in its synthesis (Appendix 2.1). It is possible to observe a number of small peaks at about 6 ppm which corresponded to impurities of unknown origin that were present in the sample. It was not possible to verify if these peaks were present in the original publication as the NMR presented is cut at 5.5 ppm. The peak at 5.13 ppm corresponds to C-1 H_a of glucosamine (Figure 2.3 (a)), an overlap between the peaks of hydrogen atoms belonging to the CH and CH₂ groups of the glucosamine and the CH₂ groups of the dendrimer is observed between 4-2.5 ppm (Figure 2.3 (b)). The peaks at 1.83, 1.10 and 0.99 ppm are thought to correspond to starting material EDC or urea which is the by-product of the reaction as well as a co-product from the reaction of EDC with water. Additional analysis of all the reagents used for the reaction was necessary to better clarify the NMR spectrum of the dendrimer glucosamine. In parallel, MS analysis using MALDI was performed to try to estimate the amount of the saccharide which had been conjugated to the dendrimer (Figure 2.4).

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Figure 2.4 MALDI analysis of dendrimer glucosamine gen. 3.5.

A 3.5 generation dendrimer has 64 available positions for loading. The highest relevant peak found in this range was 21357 Da which would correspond to 95% sugar loading. Since there is no pure compound that could be used as reference no quantitative information can be drawn from the MS analysis and it is only possible to identify a range of loading between 11 and 95%. Additionally, the generation 3.5 PAMAM dendrimer is supplied as a sodium salt in methanol, this is dried and re-suspended in water but no desalting protocol was included. Therefore, it would be expected that a value of between 10459 and 11862 Da for 0% loading could be observed due to the possible mixture of protonated and Na salt forms in the sample. Two small peaks at 10533 and 11290 were considered to represent the protonated and Na salt forms of the dendrimer without glucosamine conjugation.

In the paper by Shaunak *et al* published in 2004¹, the NMR spectra showed peaks at around 1 ppm, which corresponded to the EDC urea by-product. However, the results obtained in these experiments cannot be directly correlated since the loading of saccharide that is covalently conjugated to the dendrimer was estimated with different analytical methods. In the earlier work, the calculations for saccharide loading were made based on the NMR results. In the current work, the degree of conjugation or loading of the saccharide was estimated from results of MALDI experiments. The MALDI experiments were less accurate leading to large range for the saccharide loading (11 to 95%), in contrast to the previously reported average value of 14% obtained by NMR¹ (Appendix 2.2). The reason why the sugar loading was not estimated by NMR

here was because the spectrum was acquired with water suppression which influences the peak used as reference which corresponds to sugar C-1 H_a. However, the NMR spectrum was in agreement with the published data allowing the results to be seen as consistent. Furthermore, capillary electrophoresis analysis was performed by a collaborating laboratory at Texas Christian University, which revealed an average loading of 8-9 glucosamine molecules per dendrimer. This experiment was repeated with different dendrimer glucosamine conjugates batches always with the same averaged results (data not shown). Therefore, throughout this report an average loading of 14% corresponding to 8-9 glucosamine molecules per dendrimer will be assumed for all glucosamine dendrimer conjugates.

Analysis of the reagents involved in the synthesis of dendrimer glucosamine

All reagents involved in the synthesis, EDC, glucosamine hydrochloride and the DAB core PAMAM dendrimer were analysed both by NMR and MALDI techniques. All these reagents were obtained from commercial sources. The detailed results of the analyses of these reagents can be found in Appendix 2.1. The highlights are that both EDC and glucosamine hydrochloride did not contain impurities, though the later consists of a mixture of two anomers, α -glucosamine and β -glucosamine. The analysis of the starting PAMAM dendrimer revealed that this contained a small amount of impurities seen both in the starting dendrimer and the synthesised conjugate (Figure 2.3, peaks around 6ppm). From the ¹H-NMR spectrum, the products from the side reactions with EDC resulted in major contaminants of the final conjugated dendrimer (Figure 2.3, peaks 0.99, 1.10 and 1.83ppm). These impurities were thought to be (i) covalently linked to the dendrimer, presumably as the N-acyl isourea and (ii) non-covalently associated with the dendrimer, presumably as the urea (Figure 2.5). Based on these results it was decided to develop a new purification method to determine if the non-covalently associated impurities could be removed from the dendrimer glucosamine.



Figure 2.5 Superposition of the ¹H-NMR experiments performed on dendrimer glucosamine gen. 3.5 and the reagent EDC.

Preliminary dialysis experiments of the dendrimer glucosamine conjugate

A new batch of the dendrimer glucosamine conjugate was synthesised and dialysed using a dialysis cassette. The dialysis was conducted to see if the non-covalently associated impurities could be removed. Samples of the dendrimer glucosamine solution were taken from the dialysis cassette after 1, 17, 41 and 65 h. All samples were analysed by ¹H-NMR and the results are displayed in figure 2.6 A. The superpositioned spectra are expanded in the region where the EDC contaminant peaks appear (Figure 2.6 B).



Figure 2.6 ¹H-NMR experiment. A - ¹H-NMR spectra of the dialysed samples taken at 0h (blue); 1h (purple); 17h (green); 41h (red) and 65h (bottom blue). B – Expansion of the region between 0 and 2 ppm of spectra a.

NMR spectra in Figure 2.6 A indicate that after 1h most of the non-covalent impurities decrease significantly and after 17 hours these impurities had been removed. A closer look to the spectra (Figure 2.6 B) in the region between 0 and 2 ppm indicated the existence of peaks corresponding to the product of EDC side reactions and to the excess EDC. It is possible to observe that after 1 h one of the product forms represented by the decrease of the quintet decreases and the other represented the increase of the triplet.

After 17 h, the triplet disappears and there is a decrease on the quintet. After 41 h, no significant decrease of the quintet is observed. However, the small peak had previously been observed disappeared. After 65 h there is a decrease in the quintet but a new peak starts to appear, probably from a side reaction. Based on these results it is thought that the quintet represents the free form of EDC and that the best compromise is obtained with a dialysis time of 41 h where the EDC amount present on the sample reaches a level that can be considered acceptable trace contamination.

Additional analysis of the dialysed samples using MALDI did not provide further information. Another attempt to analyse the mixture by mass spectral analysis using electrospray failed. It was concluded that a specific column for saccharides would be necessary for ES experiments. This was not pursued further since the NMR results were conclusive and for the purposes of this work, the time investment was considered unnecessary. These new NMR results are now in accordance with the previous published results, showing an improvement in the reduction of EDC present in the sample. In the publish report the purification strategy used had reduced the urea contamination level to around 5%. No direct correlation could be made with the present work but a qualitative comparison revealed it was possible to reproduce those results with dialysis purification. Having determined the optimal dialysis time it was decided to assess the efficacy of the individual purification methods, Sephadex G25 column and dialysis, as well as their combination.

Assessment of dendrimer glucosamine purification processes

The purification method used in the previously published study utilised Sephadex G25 columns. However, it was not possible to reproduce the level of purification that was described as shown by the preliminary NMR and MALDI-TOF analyses. Dialysis was proposed as an alternative or complementary method of purification. The ideal time of dialysis and its efficacy was compared to the previous method. Ultimately all batches of dendrimer conjugates must be endotoxin free and so all required further treatment with polymixin B containing columns.

A new batch of dendrimer glucosamine conjugate was synthesised and separated in three different aliquots. One aliquot was taken and dialysed for 48 h (A) and collected for NMR analysis. A second aliquot was flushed through a Sephadex G25 (B) column and collected for NMR analysis. The last aliquot was flushed through the Sephadex G25 column and then dialysed for 48 h (C). These purified materials were then collected for NMR analysis. The results of the spectroscopic analysis are displayed in Figure 2.7.



Figure 2.7 Superposition of the ¹H-NMR spectra of the samples purified by different protocols. Blue – G25 Sephadex column; Green – 41 h dialysis only; Red – combination of G25 Sephadex column and 41 h dialysis.

Noting the region about 6 ppm (Figure 2.7), it is possible to see that the G25 column was less effective removing the impurities. This is in contrast to the samples submitted to dialysis. Moving along the spectra to the critical area where the contamination with EDC co-products are observed (0 - 2 ppm), the same situation was found. Both dialysed samples with or without the pre-treatment with the G25 column displayed a reduced amount of EDC. In contrast the aliquot which was only treated with the G25 column displayed larger amounts of the EDC co-products. Based on these results both dialysed samples were used for biological evaluation. The dialysed-only sample was submitted to cytotoxicity assays.
In vitro biological evaluation of the purified dendrimer glucosamine conjugates

The cytotoxicity of the saccharide modified dendrimers was assessed using the MTT assay. This assay is a colorimetric assay to measure the activity of enzymes that reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT (yellow) to formazan (purple)¹³⁹. This reduction takes place in the mitochondria so if a certain compound is cytotoxic it would cause mitochondrial dysfunction that would stop the reduction to formazan and consequently the purple precipitate. This precipitate is then dissolved in DMSO and the absorbance is obtained¹³⁹. The results are expressed as a percentage of survival as compared to the control. The results obtained with dendrimer glucosamine are displayed in Figure 2.8.



Figure 2.8 Graph representing the results obtained for dendrimer glucosamine gen. 3.5 for the MTT assay.

The dendrimer glucosamine conjugates showed no cytotoxicity, for the range of concentrations investigated (6.15-800 μ g/mL).

A haemolytic assay was then performed to assess the toxicity of the dendrimer glucosamine conjugates to red blood cells. The term haemolysis refers to the disruption of the membrane of red blood cells with the consequent release of haemoglobin from the cell¹⁴⁰. A haemolytic assay aims to assess if a certain compound has a toxic effect on red

blood cells. Haemolytic compounds do not generally make good medicines. The results are usually displayed in relation to a positive control (e.g. Amphotericin-B) which has a haemolytic effect (Figure 2.9). No haemolytic activity was observed for dendrimer glucosamine gen. 3.5.



Figure 2.9 Haemolysis assay with the results for dendrimer glucosamine.

The effect of the purified dendrimer glucosamine on the production of cytokines and chemokines was then assessed by incubating cells with both the dendrimer glucosamine and LPS. The LPS was used to induce cytokine production. The dendrimer glucosamine conjugate was evaluated to determine if cytokine production could be inhibited after exposure to LPS. For real time RT-PCR analysis, the house keeping gene HPRT was used as reference to normalise the results. The results obtained for the cytokines (TNF α , IL1 β and IL6) and chemokines (MIP-1 β and IL8) that were studied are displayed below.



Figure 2.10 Inhibitory effect of dendrimer glucosamine on the release of cytokines TNF- α ; IL1b and IL6. The first column shows the results for dendrimer glucosamine purified with 41h dialysis only (referenced as A2) and the second column the results for the dendrimer glucosamine purified by the G25 Sephadex column followed by 41 h dialysis (referenced as A3).

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The results (Figure 2.10) show that dendrimer glucosamine inhibited cytokine production. Using the purified, dialysed samples, the number of mRNA copies for all of the cytokines that were tested significantly decreased to near control levels.



Figure 2.11 Inhibitory effect of dendrimer glucosamine on the release of the chemokines MIP-1b and IL8. The first column shows the results for dendrimer glucosamine purified with 41 h dialysis (referenced as A2) and the second column the results for the dendrimer glucosamine purified by the G25 Sephadex column followed by 41h dialysis (referenced as A3).

The results for chemokine release were similar to those found for the cytokines (Figure 2.11). There is a significant decrease of chemokine release and, particularly, a concentration response was observed for IL8. These results were essentially the same as those published¹.

Based on these results, it was concluded that 41 h dialysis was a suitable method for the purification of dendrimer glucosamine gen. 3.5 and that it should be used for further studies involving this molecule. Neither the purification method used for dendrimer glucosamine synthesis nor the different core of the dendrimer seem to have an impact on its inhibitory effect compared to the previously published data¹.

Characterisation and biological evaluation of the generation 3.5 PAMAM dendrimer modified with 2-Acetamido-2-deoxy-b-D-glucopyranosyl amine

A new dendrimer modified with saccharide was then prepared. This conjugate was designed to display a saccharide that was closer to the one present on Shigella LPS. Lipid A is a conserved region of LPS in Gram negative bacteria, it consists of a phosphorylated N-acetylglucosamine dimer and 6 or 7 lipid chains⁵³. It was rationalized that using a saccharide molecule that resembled the conserved residues on Lipid A might enhance the activity of the dendrimer conjugates and give more insight into the interactions with the biological target responsible for inhibiting cytokine release.

After initial experiments, the previously described protocol was changed. The same volume of EDC used for the glucosamine conjugates synthesis was added in small fractions throughout the reaction time, since the reaction of EDC with water competes with the activation of the carboxylic acid groups. The second step in the synthesis of the dendrimer conjugates is the nucleophilic attack by the amino group of the saccharide. In contrast to glucosamine, in N-acetyl glucosamine analogue the nucleophilic amine is bound to the carbon adjacent to the anomeric carbon (Figure 2.12). The amine is less reactive due to the electronegativity of the oxygen and consequently the reaction is slower. The reaction of EDC with water becomes competitive. Adding EDC in the same amount but at different time points of the reaction allows better availability of EDC for carboxylic acid activation.



Figure 2.12 2D Structure of 2-Acetamido-2-deoxy- β -D-glucopyranosyl amine. The red arrow shows the amino group responsible for the nucleophilic attack to the carbodiimide activated terminal carboxylic group of the PAMAM dendrimer.

The ¹H-NMR analysis of the N-acetyl glucosamine dendrimer revealed the presence of impurities. A 41h dialysis was performed in an attempt to reduce the impurities to trace levels. It was possible to observe a small number of peaks, around 6 ppm corresponding to impurities present in the sample. These peaks disappeared after dialysis. At around 5 ppm a peak corresponding to C-1 H_{α} of the saccharide was observed corroborating the formation of the covalent bond to the dendrimer. This peak did not disappear upon dialysis.

The cytotoxicity of the new dendrimer saccharide was investigated with a MTT assay as done for the glucosamine dendrimers (Figure 2.13).



Figure 2.13 MTT assay obtained for dendrimer 2-Acetamido-2-deoxy-b-D-glucopyranosyl amine conjugate.

The value obtained for the 50% lethal dose (LD₅₀) of the N-acetyl glucosamine dendrimer analogue was 335ug/ml. The concentration range used for monitoring the levels of chemokine and cytokine expression was from 25 μ g/mL to 200 μ g/mL, and always inferior to the LD₅₀.

Finally, the in-vitro biological properties to inhibit cytokine release after induction with bacterial LPS showed that these new dendrimer saccharides could inhibit cytokine release (Figure 2.14).



Figure 2.14 Inhibitory effect of dendrimer modified with 2-Acetamido-2-deoxy-b-Dglucopyranosyl amine on the release of the: Top - chemokines MIP-1b and IL8; Bottom - cytokines TNF- α and IL6.

The expression of the chemokines (Figure 2.14 top) and cytokines (Figure 2.14 bottom) tested was shown to decrease significantly in the presence of the new dendrimer conjugate (Figure 2.14) to levels near the control levels.

These latter results suggest that the interaction between the saccharide modified dendrimers and the biological target is not limited to just glucosamine but includes Nacetyl glucosamine. Preliminary data also from this lab and collaborator labs also shows that di-glucosamine (chitobiose) when incorporated into dendrimers also has an antiinflammatory effect. In contrast, previous studies had shown that the generation 3.5 PAMAM dendrimers without any modification did not have any biological activity¹⁰⁷. While the studies were in progress, triazine dendrimers were synthesised and modified with glucosamine by collaborators at Imperial College London and Texas Christian University. No biological activity was found. This in turn may suggest that structural differences of surface modifications may not necessarily be the key factor in determining biological activities, but that molecular properties such as overall charge distribution and hydrophobicity may play key roles. This is an important result for this project, as one of its aims is to understand the interaction between the dendrimer and the biological target. However, since no improvements have been achieved a key decision was made based on the cost, chemical properties or biological relevance of this new dendrimer: this route of optimizing the surface modifications of PAMAM dendrimers was not pursued further. The efforts were focused on the use of molecular modelling to gain better understanding of the molecular properties and biological relevance of these glycosylated dendrimers with immuno-modulatory properties and use this knowledge to rationally design novel dendrimer.

Conclusion

The synthetic route used to prepare the dendrimer glucosamine conjugates minimises endotoxin contamination. Although the synthetic route was relatively time and cost effective, the divergent approach results in a mixture of conjugates in terms of regiochemistry and loading. This synthesis does not yield a defined molecule, especially since it is known that PAMAM dendrimers display defects in their structure. Characterisation was primarily used to ensure the conjugates were free of urea impurities and had minimal amounts of acylisourea products. It was found that a 41 h dialysis period was sufficient to remove the EDC derived species. It reduced the amount of EDC products to trace levels without decreasing the *in vitro* biological activity of the glucosamine dendrimer conjugates

A new dendrimer conjugate was synthesised, with 2-acetamido-2-deoxy- β -D-glucopyranosyl amine conjugated to it surface carboxylates. The saccharide is a derivative of N-acetyl-glucosamine, which is present on the *Shigella* LPS that is known to trigger the TLR4 mediated inflammatory response. This new dendrimer saccharide conjugate proved to be biologically active against the LPS triggered inflammatory response. Since no improvements were observed compared to the glucosamine modified dendrimer, further examination of this new conjugate was not pursued. The new synthesis did however indicate that the interaction between these glycosylated dendrimers and their biological target might lack some degree of specificity. It is acknowledged that the starting PAMAM dendrimers are not active as well as another class of dendrimers which are described in chapter VI of this thesis.

These results were important for the overall project as they allowed a better understanding of the dendrimeric system and its heterogeneity. Furthermore, they revealed important information towards the interaction of the dendrimer PAMAM structure with the biological target.

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CHAPTER III - COMPUTATIONALLY GENERATED 3D STRUCTURES OF SACCHARIDE MODIFIED DENDRIMERS

Introduction

Dendrimers are hyper-branched macromolecules with polymeric nature. The increasing interest in these molecules and their applications in various areas has raised the question of an appropriate nomenclature capable of accurately describing their topology and structure. To start with, the naming and description of the 2D structure (topology) of these molecules is still not universal. IUPAC nomenclature is generally acceptable way of naming molecules and should enable the exact description of any type of a molecule. Though this can be applied to dendrimeric molecules¹⁴¹, as dendrimer structures are complex and ramified, this type of nomenclature becomes less clear with increasing size and does not capture all features of the structures. The same can be said for the Nodal nomenclature, which despite being able to describe dendrimers, has not been widely used^{142, 143}.

The two main nomenclatures developed for dendrimeric structures are Newkomenomenclature¹⁴⁴ and cascadane¹⁴⁵. Both of these nomenclatures are capable of representing the dendrimers hyperbranched nature and make use of the repetitive units that constitute dendrimers to simplify their notation^{144, 145}. However, as the molecules become bigger the notation becomes complex and difficult to interpret. Further simplification on dendrimer nomenclature, taking advantage of their repetitive topology and symmetry has been proposed with a dotted cap notation¹⁴³. This notation represents dendrimers as building blocks, with a core unit, monomers and capping groups, the core is bound to the monomers forming the dendrimer framework where the caps are attached¹⁴³. The dotted cap notation then represents the surface of the dendrimer by means of sequential caps (Figure 3.1).



Figure 3.1 Example of the dotted cap notation for a polylysine dendrimer¹⁴³. In the image 27 represents the whole dendrimer structure; the core is noted as 28, branches as 29 and the caps B, Z and F. Adapted from Roberts *et al*¹⁴³.

In this chapter the aim is not to define a nomenclature for dendrimeric structures but to develop an efficient method to generate three dimensional structures of these molecules from a linear sequence, which to the best of my knowledge has never been done. Nonetheless, in this work the same building blocks concept and linear sequence assembly as defined in the dotted cap notation was used, using as model system a generation 3.5 PAMAM dendrimer modified with glucosamine. A closer look to the dendrimer structure reveals that though these are complex hyperbranched structures, in reality, can be described with four different types of subunits, core, branches, surface and surface modification (Figure 3.2).



Figure 3.2 Two dimensional structure of a generation 3.5 PAMAM dendrimer modified with glucosamine, coloured based on the units that make up its structure. Black – core; Green – branches; Blue – surface; Red – glucosamine. Adapted from Shaunak *et al.*¹.

In an equivalent manner as in the dotted cap notation the dendrimers can be represented by a linear sequence of monomers, as shown in Figure 3.3.



Figure 3.3 Translation of the dendrimer glucosamine topology into sequence of monomers. Four monomers (A-D) have been defined as being necessary to define the PAMAM structure.

This type of representation and structure generation has been widely used for protein assembly, having amino acids as monomers and nucleic acids with their nucleotides.

In fact, there is a wide choice of software capable of generating starting 3D structures from linear sequences, though most of them are dedicated to proteins and more recently to nucleic acids. It was thought that the repetitive structure of dendrimers represented a good parallel with the protein structure and that the possibility of versatile method of generation of hyperbranched dendrimers from a linear sequence would open doors for the use of molecular modelling tools to the study of dendrimeric structures.

Two main packages dedicated to sequential assembly of molecules are Gromacs¹⁴⁶ and XPLOR¹⁴⁷, being the first mostly used for dynamic simulation of proteins and the second for structure generation based on NMR and X-ray experimental data. Regardless, of the package, initial monomers have to be defined within a topology and a parameter files, where all the information regarding each individual atom and how they are brought together first in monomers and further along as a molecule, has to be described. Topology and parameter files are specific to each force field. Molecular mechanics methods have to be used for parameterisation and force field development. Molecular mechanics methods are based on empirical methods and those are based on the classical laws of physics to predict chemical properties. They neglect explicit treatment of electrons, not being able to deal with problems where electronic and quantum effects

prevail, like bond formation or breaking¹⁰⁹. The potential energy of the system under study is determined using force fields¹²⁵.

Molecular mechanics techniques treat atoms within molecules as variable masses connected by springs and states that the coordinates of these atoms are possible to determine in the place where the springs are in equilibrium¹⁰⁹. In reality, these atoms are subject to a combination of repulsion and attraction forces, these contributions form the basis of force fields¹²⁵. Force fields allow the determination of the energy of a system by calculating all the forces influencing the system. To be able to create a force field, it is necessary to derive an equation for each of the energy terms as well as any parameters essential for the equations¹⁰⁹. In general terms, the total energy can be defined as:

$$E_{Total} = E_{bonded} + E_{non \ bonded}$$

Where each term can be further decomposed into,

$$E_{bonded} = E_{bond} + E_{angle} + E_{torsion}$$
$$E_{non \ bonded} = E_{electrostatic} + E_{van \ der \ Waals}$$

The specific determination of each term of the above equations is specific to the force field used but if for instance bonds are treated as springs, it becomes necessary to know the spring constant and the equilibrium distance between every two atoms in the system¹⁰⁹.

All the equations come from classical physics, and the parameters from experimental data, or from higher level quantum mechanics calculations. Some of the essential energy terms are bond length or bond stretching, bond angles or bond angle bending, torsion angle or dihedral angle rotation, van der Waals forces, hydrogen bonding and electrostatic interactions¹²⁵. The most widely used force fields are AMBER, CHARMM, MMx and OPLS.

In summary, this chapter describes the development of an *in silico* method that allows the generation of 3D models of these dendrimers. XPLOR-NIH 2.18 was used to generate the 3D models from a starting sequence and for its initial refinement. Force fields CHARMM22 and CHARMM27 were chosen in this study, since they have been

continuously developed and the extensive work done with these force fields provides a great diversity of atom types and parameters, helpful for the development of novel monomers in macromolecules¹⁴⁸.

Experimental Section

Monomer assignment

The morphology of the dendrimer allows the structure to be seen as a sequence of interconnected monomers despite their branched nature. Four main monomers were defined in order to sequentially build the dendrimer structure (Figure 3.3); one diaminobutane core monomer (DAB; A), one monomer constituting the repetitive branch subunit (DBB; B), one monomer representing the carboxylated surface (DBC; C) and a monomer for glucosamine (DBD; D) (Figure 3.4). The hyper branched structure of Starburst PAMAM dendrimers in this work is derived from a diaminobutane core, as a model. Alternative core units were also defined and can be found in Appendix 3.1.

Dendrimer Block A - DAB

Dendrimer Block B - DBB

HN-C

Dendrimer Block C - DBC

Dendrimer Block D - DBD



Figure 3.4 Monomers assigned for the dendrimer glucosamine used as a model for the development of 3D dendrimer structures. Additional monomers can be found in Appendix 3.1.

Atom types definition

The atom types necessary to describe dendrimer monomers were defined by comparing the nature of the atoms in the structure and its environment with the ones already present in CHARMM22 and CHARMM27 force field. New names were given to the atom types with the purpose of simplifying further work with the force field parameters. However, not all atoms present in the dendrimer structure could be assigned based on similarity, and in these cases, new atom types were defined (examples will be provided in the results section).

Parameter data generation

The parameter file of the CHARMM force field contains values of all bond lengths, angles, dihedrals, improper dihedrals and non-bonded parameters and the constants associated with these defined in terms of atom type and not atom name.

The values were grouped according to atom types and the average value determined and registered in the parameter file. The constants for bond length, bond angle and dihedrals as well as the non bonded parameter for each atom type were assigned based on similarity with atom types from previously developed CHARMM force field parameter files. The files were written according to CHARMM22 and CHARMM27 syntax.

The values for bond length, bond angles and torsion angles were obtained from the lowest energy structure of a gen. 2.5 glucosamine resulting from a Monte Carlo conformational search. An initial structure of the 2.5 generation glucosamine dendrimer was built in Maestro¹⁴⁹. The first step was the determination of the protonation states of ionisable groups at the target pH set to 7 using Ligprep 2.2¹⁴⁹ and OPLS-2005 force field¹⁵⁰. Macromodel 9.6¹⁵¹ was used for conformational search using OPLS_2005 force field and implicitly defined model for water as solvent. The option to distinguish enantiomers was used to preserve defined stereochemistry. The defaults cut off values were 8 Å for Van der Waals interactions, 20 Å or electrostatics and 4 Å for H-bonds. Additionally, 2500 iteration steps were used to optimize 1000 generated conformations. Different conformations within an energy window of 5 KJ/mol were saved.

The output files were saved as files with .mae extension and imported to Chem Bio3D (ChemBioOffice 2008, CambrigeSoft) where a table containing all the bond lengths, bond angles and torsion angles was generated. This table was then exported to Microsoft Excel (Microsoft Office) and saved for further analysis.

Dimeric combinations of the monomeric units defined for dendrimer glucosamine were built in Maestro¹⁴⁹. The protonation state was determined using Ligprep 2.2¹⁴⁹ and conformational search was performed with Macromodel 9.6¹⁵¹, as described above. The output files were saved as .mae for further calculations.

Further structure optimization at higher theory level was performed with Jaguar 7.5¹⁴⁹ using HF/6-31G* method, selecting water as a solvent with SM6/PBF algorithm. The properties calculated were electrostatic surface potential (ESP) charges and surfaces, orbitals saved for visualization were HOMO and LUMO and their two neighbouring occupied and unoccupied, respectively. The output files were saved as .mae and the charges analyzed manually with Maestro measuring tool menu.

Initially, the dihedral values used for the monomeric units of dendrimer glucosamine were determined using the same method as for bond length or angles; however, these values did not correspond to the syntax used by the force field chosen. The new values were assigned according to Table 3.1.

Table 3.1 CHARMM dihedral parameters used for dihedrals assignments of the dendrimer monomers (http://www.ks.uiuc.edu/Training/Tutorials/science/forcefield-tutorial/forcefield-html/node6.html).

Dihedral Parameters in CHARMM format			
Multiplicity, n	Phase, δ	Location of Minima	Notes
1	0	180	Yields trans conformation
1	180	0	Yields cis conformation
2	0	90, 270	
2	180	0, 180	Useful for enforcing planarity
3	0	60, 180, 300	Emphasises staggered conformation of
			sp3 carbons
3	180	0, 120, 240	Emphasises eclipsed conformation of
			sp3 carbons

Sequence based generation of 3D dendrimer structures and molecular dynamics simulation

The 3D structure generation was carried out using XPLOR-NIH 2.18 (http://nmr.cit.nih.gov/xplor-nih). The generate.inp is used to generate psf file utilizing the topology file and the sequence of monomers. A reference number for the patch statement was attributed to each monomer of the sequence. The generated psf file was used as an input for random.inp file, where random coordinates for all atoms were generated based on the previously defined parameters and a coordinate file (.pdb) was saved. Initial optimization of atom coordinates was achieved by a loop of 500 steps simulation steps with increasing contributions of Van der Waals and decreasing the temperature from 1500K to 300K. This set of simulation was followed by 5000 steps of minimization.

Resulting pdb and psf files can be utilized for further simulation using XPLOR-NIH or NAMD¹⁵². Additionally, these output files can be either visualized or converted into other file formats by VMD¹⁵³ or OpenBabel¹⁵⁴. Bond orders or atom types have to be checked after conversion.

Refine structures with XPLOR-NIH

Two different experiments were submitted at this point, one with explicit solvation and another without solvation. For the solvation experiment it was necessary to create a PSF and a PDB file containing both the dendrimer and water. This is achieved by running a mkpsf type file in XPLOR-NIH where the force field parameter and topology files were added. The simulations with and without solvation were performed with the simulation annealing protocol (SA) from XPLOR. Fifty structures were generated as pdb files. The initial annealing temperature was set to 1000K with 2000 cooling steps to a final temperature of 100K. The nonbonded interactions were set as 4.5 Å with a tolerance of 0.5 Å. The generated structures were minimized by 200 steps using the Powell algorithm¹⁵⁵.

Solvation and simulation with NAMD

In addition to the efforts to use Xplor for molecular dynamics simulation, parallel efforts were also made to utilise NAMD. For this purpose, CHARMM22 topology and parameter files were rewritten using CHARMM27 syntax. Using VMD and the TK console, the dendrimer was explicitly solvated. The TK console was also used to obtain the coordinates of the cell used to simulate the dendrimer. Taking as a reference the online tutorials (http://www.ks.uiuc.edu/Training/Tutorials/), different scripts for minimization and equilibration and simulation were written. The NAMD molecular dynamics program¹⁵⁶ version 2.5 was used with the CHARMM27 force-field parameters¹⁴⁸ to simulate a cell of 120 Å in all three dimensions (x, y, z). The cell was generated with the origin coordinates set to (-2, -1, 2.8). For minimization and equilibration purposes, the temperature was maintained at 310 K and pressure at 1 atm by means of Langevin dynamics using a collision frequency of 5/ps. The van der Waals interactions were switched smoothly to zero over the region 12-13.5 Å and long-range electrostatic interactions were treated with the Particle Mesh Ewald (PME) method¹³³ with a PME Grid of 120 Å in all dimensions. This equilibration simulation was run for 2500 time steps of 1 fs followed by additional 100 minimization steps.

For the production simulation, a similar script was developed using the same software and the CHARMM27 force-field parameters to simulate a cell of 120 Å in all

three dimensions (x, y, z). It was generated with the origin coordinates of (-2, -1, 2.8). For simulation purposes, the defined cell was made flexible though shape was fixed along the XY plane, to keep the structure within the cell. The temperature was maintained at 310 K and pressure at 1 atm using Langevin dynamics with a collision frequency of 1ps. The van der Waals interactions were switched smoothly to zero over the region 10-11.5 Å and long-range electrostatic interactions were treated with the PME method with a PME Grid of 120 Å in all dimensions. This script was run for 20,000 time steps of 1 fs. VMD¹⁵³ graphical user interface was used to visualise the results from the molecular dynamics simulations with NAMD.

Results and Discussion

To generate 3D structures of the PAMAM dendrimers from the sequence required that different software packages were considered for method development and parameterisation. Gromacs¹⁴⁶ and Xplor-NIH were the key packages that were identified with the highest potential to be used for the PAMAM dendrimers. Initial efforts were made with Gromacs since this software can generate a 3D structure from sequence without knowing initial coordinates and also perform molecular dynamics simulations. The use of a single software package would ensure that there was consistency in the parameters and compatibility with the dendrimeric system throughout the study. However, Gromacs would only allow patching two sequential monomers to each other, which for hyperbranched structures like PAMAM dendrimers proven to be impractical for a simple assembly from the sequence of dendrimer. Although the main function of Xplor-NIH is not to run molecular dynamics simulations without experimental constraints it is compatible with NAMD and so it also seemed like a suitable choice since there is the possibility to apply more than one patch between nonsequential residues.

Monomer units were initially defined for the glucosamine modified dendrimers (Figure 3.4, experimental section). The geometry and electronic properties of the monomers within the dendrimer are not known due to the absence of experimental structural data for this dendrimer type. A set of *ab initio* and molecular mechanics calculations was conducted on various fragments of the dendrimers to evaluate geometric parameters of a 3D structure. The resulting values were used for the development of the different force field parameters. CHARMM22 and CHARMM27 force fields were used as reference for the atom type assignment¹⁴⁸.

Topology and parameter files development

Most force field topology and parameter files found in the literature have been developed for proteins using amino acids as the monomeric units¹⁵⁷. More recently, force field development has been done for nucleic acids¹⁵⁸, saccharides¹⁵⁹ and lipid

molecules¹⁶⁰. The starting point for atom type assignment was to look for similarity between the new dendrimer monomers and analogous monomer that had been previously developed for proteins, nucleic acids and lipids.

When looking at the dendrimer's monomers it is possible to identify some protein like atom types (Figure 3.4, experimental section). For example in monomer B carbonyl carbon in this monomer is similar the carbonyl carbon present in some amino acids such as aspartic acid, asparagine or glutamine. In the saccharide monomer (DBD), the nitrogen resembles nitrogen in a peptide bond; all other atoms in this monomer are equivalent to the ones found online in the CHARMM22 saccharide parameter file¹⁴⁸.

These parameters were developed for the purpose of creating a versatile set of tools that allowed the quick generation of 3D models for dendrimers. Thus different cores were tested (Appendix 3.1) and the parameters for these were assigned so that independent of the size of the core chain, the same atom types could be used and different dendrimers can be created. Using a diaminobutane core as a model, the atoms in the chain were assigned different atom types according to their distance from the terminal nitrogen. Those closest to the NH₃ were assigned as generic aliphatic sp3 for CH₂. All others were assigned as described in a file, found online, specific for lipid chains¹⁴⁸. Their charges in the topology file are not significantly different. However, in the parameter files, this distinction allows the definition of different dihedrals.

Nevertheless, some of the atoms, particularly after the conjugation of the monomers (i.e. patch), present a unique environment. Therefore it was necessary to define novel atom types for these monomers (Figure 3.5 (a)). This is the case for all of the terminal nitrogens from the core and branch monomers (DAB, DBB). Figure 3.5 (c) shows how the nitrogen atom, in both monomers, acquires a tetrahedral conformation not present in the force field files. In this case, the nitrogen was assigned as amide nitrogen. A different name was given (from the generic CHARMM22 force field) to allow the attribution of different bond lengths, angles and dihedrals to this case. Another example of this is the oxygen atom on the surface monomer (DBC). Although in Scheme 3.2 this is represented as protonated, when Ligprep was run establishing 7 as the target pH, the oxygen atom was deprotonated in solution. After patch, this atom acquired a carboxylate

oxygen type configuration. This situation is not found in any of the reference files of Chamm22. Thus a new atom type was established and fully parameterised.

Ab initio methods are based on quantum chemistry principles. They aim to determine potential energy values by solving Schrödinger's equation¹¹⁰. The Hartree Fock theory and 6-31G* basis set were used in this study. These allow the wave function to be solved using numerical methods; however, they also constitute limits to the calculations^{111, 115}. A molecule with the dimension of these dendrimers would involve a large number of terms that the computing time necessary to determine the potential energy value would not be sustainable. The application of these methods for calculations of individual monomers would be possible. However, all the information about the bonds connecting the monomers within the dendrimer and the properties changes of the atoms involved in bond formation would be lost. Thus, a variety of computational methods were used to parameterise combination of monomers fully.

The combinations of the monomers representing fragments of the dendrimer structure were designed in Maestro and a conformational search followed by optimization with Jaguar was performed (Figure 3.5)



Figure 3.5 Monomer combinations submitted to *ab initio* calculations for determination of atom charges. A - Monomer DBB bound to two DBCs represent the unmodified surface of the dendrimer under study; B - Monomer DBC bound to DBD represents the glycosylated surface; C - Monomer DAB bound to two CH3 groups on one side and two DBB represents the core of the dendrimer; D - Three monomers of DBB bound together represent the branch structure of the dendrimers under study.

To understand the geometry of monomers and patches within the large molecule environment, bonds length, angles and torsion angles were measured from a generation 2.5 dendrimer glucosamine that had been submitted to Conformational Search in Maestro (Figure 3.6). Generation 2.5 was the largest molecule representing all the environments in the dendrimer, which could be studied by the conformational search method implemented in the Macromodel.



Figure 3.6 Lowest energy structure of a dendrimer glucosamine gen. 2.5 obtained after conformational search with Macromodel. This structure was used for the assignment of bond length, bond and torsion angles between the monomers.

Topology files contain information on the atom types, masses and charges of each atom in a monomer and also the information needed for applying patches between residues so that a PSF (protein structure file) file can be created. In the beginning of the file, a list of atom types and the corresponding masses are listed followed by a description of each monomer. To each atom in the residue a name, type and charge is given, and the connections between them are described. Finally, the values of internal coordinates (IC) were described for each pair of four consecutive atoms. Although these IC values are not essential, these were added to allow calculation of the missing atom coordinates based on coordinates of atoms with known positions. The IC values were assigned based on a conformational search that was performed on the generation 2.5 dendrimer glucosamine and the constant values were based on similarity with the reference files¹⁴⁸. The importance of adding these values relies on the fact that the structure will be generated by a random method. Since there are no experimental structural data, these constraints are useful to ensure that the correct stereochemistry is achieved, unlike the parameters in the force field parameter file the internal coordinates are not relative to Cartesian space but define bonds, angles and torsions of an atom relation to one that precedes it.

Additionally, information concerning the patch of monomers is also present in this file. All new connections between residues have to be stated for the PSF file as well as the ones that are removed. Alterations of atom types and charges should also be added so the correct parameters are given for the PDB (protein data bank) file. The complete topology file can be found in Appendix 3.2.

In the parameter file, all descriptors are registered in terms of atom types. For bonds, each entry contains a pair of atoms, a spring constant and an equilibrium length. The formula for the bond potential function is $V = K_b (b - b_0)^2$. For the dendrimer assignments, the value of the constants (Kb) were based on the reference files¹⁴⁸ and the equilibrium lengths were based on the conformational search performed on the gen. 2.5 dendrimer glucosamine.

The second section in the parameter file lists the bond angles, where all possible combinations of the three atom types are registered. Each entry consisted of three atom types, a spring constant, and an equilibrium angle. The potential function for bond angles was defined by the equation $V = K_{\theta} (\theta - \theta_0)^2$, where K_{θ} represented the spring constant and θ the bond angle.

The dihedral parameter plays a fundamental role in the structure because it determines the conformations allowed for the molecules under study. This was one of the most problematic parameters to address. Since there was no structural data available of the PAMAM dendrimers, it was not possible to add nOe constraints or other experimental based restrictions during the random generation process. Thus the initial

geometry arrangement of atoms of molecules and achieving desired stereochemistry relies to a significant degree on the set of dihedral assignments.

The dihedral values were written as shown in Table 3.1 using the following link as reference: (http://www.ks.uiuc.edu/Training/Tutorials/science/forcefield-tutorial/forcefield-html/node6.html) and according to the torsional angles observed in the generation 2.5 dendrimer glucosamine (Figure 3.6).

For the saccharide monomers, this was not followed. A saccharide force field file that was available online was taken as reference saccharide¹⁴⁸. All the dihedrals that were added were dummy dihedrals. This means that all constant values were set to zero. In this way, the simulation software will not ask for them when generating a molecule and there will be no constraints on setting torsion angles. Additionally, and in the absence of dihedrals, the necessary improper dihedrals defined for non consecutive atoms were added.

For the core monomer, different atom types were defined so that lipid like dihedrals could be added. In case of long chain cores, such as dodecane core, the generation of structures led to possibly higher energy eclipsed conformations as opposed to expected staggered arrangement of CH_2 groups. This could be corrected only with the use of parameters that are typically utilised with lipids. In specific cases like in monomer DBB (Figure 3.4), it was necessary to add improper dihedrals (see parameter file in appendix) to maintain the planarity of the O-C-N that resembles a peptide bond.

The last section of the parameter file concerns the nonbonded interactions term. This is specific for each atom and the values of the reference files were always used. The complete parameter file can be found in Appendix 3.3.

Both parameter and topology files were initially written with the syntax of CHARMM22 force field because this force field is recognised by Xplor.

Generating the structure with Xplor

The second step in the study was the generation of the dendrimer glucosamine, used as a model for the method development, with Xplor-NIH. As mentioned, this was a three step process, where PSF file generation (Figure 3.7) was followed by generating a set of

PDB coordinate files using short simulation and minimisation (Figure 3.8) and finally the structures were refined using a simulated annealing protocol.



Figure 3.7 Scheme representing the sequence of actions executed by Xplor-NIH with generate.inp file (complete generate.inp file can be found in Appendix 3.4).

- Reads parameter file

- Reads psf file previously generated

- Reads nOe file
- Defines dimensions of the grid through vectors
- Defines energy terms
- Generating random coordinates for all atoms

- Stage 1 loop:

- 100 steps dynamics verlet

- vdw contribution = 0

- time step = 0.04 fs
- non bonded cut off = 100 Å

- Stage 2 loop

- 500 steps dynamics verlet

- vdw contribution = 0.002 to 0.005

- time step = 0.003 fs

- non bonded cut off = 4.5 Å
- bath Temperature = 1500 K

- Stage 3 loop

- 2000 steps dynamics verlet
- vdw contribution = 0.01
 - time step = 0.003 fs
- bath Temperature from 1500 K to 300 K

- Powell Minimization

- 5000 steps

- Saving a pdb file

Figure 3.8 Scheme representing the sequential generation process executed by Xplor-NIH with random.inp file (Complete rand.inp file can be found in Appendix 3.5). As it can be seen in Figure 3.8, during the generation of the random coordinates of the glucosamine dendrimer structure, the contribution of the nonbonded parameters was slowly increased throughout the protocol. This was done so that the atoms can pass through each other during the protocol and adopt reasonable conformations. In order to obtain an extended structure and to ensure a common starting point for all of the structures that were generated, a nOe distance file was written and taken into consideration in the beginning of the generation.inp process. A final minimisation was then performed so that the most stable structures could be generated and saved. In this study 10 structures were written and saved for further calculations.



Figure 3.9 Example of a structure of the dendrimer glucosamine generation 3.5 generated with random protocol from Xplor-NIH described above.

Figure 3.9 displays one of the structures obtained with Xplor. Detailed observation reveals an unexpected folding of the branches, with some carboxylic end groups folding

towards the core of the dendrimer. This potentially unfavourable folding can be related with one of the draw backs of this method which is the electrostatic forces were ignored during the process of generating the structure. Several attempts were made using different gradients of electrostatic contribution over the loop, as performed to determine the Van der Waals interactions. However, none of the attempts was successful; either the structure fell apart or the torsion angles were corrupted resulting in high energy structures. The first step where electrostatic interactions were taken successfully into account was when the molecule was solvated in a water box.

Further simulation with Xplor was performed using the simulating annealing protocol (Figure 3.10 A). In the resulting structure the water molecules are not contained in the box with the dendrimer but are dispersing. This phenomenon is a result of the absence of periodic boundary conditions method in the Xplor-NIH. In Figure 3.10 B, the results of the SA protocol without solvation are displayed, where no structural problems were detected (complete refine.inp file can be found in Appendix 3.6).



Figure 3.10 Structures obtained with the simulating annealing protocol with Xplor-NIH. A - Structure solvated prior to simulation; B - Non-solvated molecule.

All ligand-receptor interactions occur in an aqueous environment and the presence of water can cause changes in the properties and conformation of these molecules. For this reason, simulating the generated structures without implicit or explicit solvent representation can lead to unreliable results during docking. However, some of the software packages available (e.g. GRID protocol) allow the generation of different surfaces for the dendrimer by taking into account hydrophobic and ionic effects.

Although it was not possible to solvate the dendrimer conjugates with Xplor, this can be achieved at a later stage. NAMD software package was used to simulate the generated dendrimer conjugates. In order to use this software both topology and parameter files, previously written with CHARMM22 syntax, had to be converted into CHARMM27 syntax, to be read by the NAMD.

Unlike what had happened with Xplor-NIH, the results obtained with NAMD show the water molecules distributed all over the box, including between branches and close to the dendrimer's core (Figure 3.11).



Figure 3.11 Dendrimer glucosamine generation 3.5 solvated with NAMD protocol.

Before the molecular dynamics simulation of the dendrimer glucosamine structures, these were solvated and then submitted to an equilibration protocol at 310K followed by minimisation, with NAMD. This protocol still does not take into account the electrostatics apart from the interaction with the water molecules. This may be a reason why the saccharide molecules seem to be pointing inwards (Figure 3.12).



Figure 3.12 Detail of dendrimer glucosamine after solvation with NAMD, showing the positioning of the saccharides present (represented in CPK).

However, the dendrimer conjugates are known to have a higher number of glucosamine molecules on their surface¹. As such, further computational experiments were needed with a higher number of saccharide molecules on the dendrimer surface.

These structures were then submitted to a 20 ps simulation protocol with NAMD and electrostatic interactions were slowly introduced (Figure 3.13).


Figure 3.13 Dendrimer glucosamine generation 3.5 after equilibration and minimisation with NAMD software package.

The results from the equilibration and minimisation step were visualised with VMD graphical user interface (GUI). Even though these files can be read using this software, the simulation script did not run because the previous files could not be read. Further attempts for simulations with NAMD were performed without success. At the time of these studies, Desmond (Schrödinger) was made available for academics. Desmond recognized the dendrimer files and maintained the stereochemistry that was defined with Xplor-NIH without requiring any further changes on the force field files (i.e. translation from 2.2 into charmm27). At this stage it was then decided to use the structures generated by the SA protocol of Xplor-NIH directly as an input for all molecular dynamics simulation studies using Desmond.

Conclusion

A method to generate 3D models from the sequence of saccharide-modified dendrimers was established. The generation of these models constitute a fundamental step for the study of the effect of glycosylation on dendrimer structure and for the study of the interactions of the saccharide conjugate dendrimers with the LPS recognition system. At this stage, the generated structures cannot be considered fully representative because a long simulation in a water environment that includes electrostatic contributions is also required. However, a new software package, Desmond, was found capable to successfully carry out molecular dynamics simulations in the dendrimeric structures, overcoming the solvation problems of these molecules and allowing for further structural studies on PAMAM dendrimers.

CHAPTER IV – LOADING AND DISTRIBUTION OF GLUCOSAMINE MOLECULES ON THE DENDRIMER SURFACE AND DYNAMIC BEHAVIOUR ANALYSIS

Introduction

In chapter II it was demonstrated that the saccharide loading of these dendrimers can be estimated but the position and number of units on these molecules cannot be determined experimentally since the synthesis route results in the random conjugation of saccharide to the dendrimer carboxylates. The current divergent synthetic strategy for these saccharide modified PAMAM dendrimers does not allow for control of the number and positioning of the saccharide molecules conjugated. Hence it is not possible to determine detailed structure-activity correlations. The possibility of conducting a convergent synthetic method was pondered; however, it was decided to assess the impact of the loading and positioning of the saccharides prior to embrace an expensive and time consuming new synthesis strategy. A method that allows the generation of 3D models of these dendrimers *in silico* has been developed and presented in chapter III, allowing the use of molecular modelling techniques to gain an insight about the loading and assessment of their dynamic behaviour that can contribute for a better understanding of the biological relevance of these molecules.

Reactivity of molecules can be estimated by understanding their electronic properties. In 1981, Fukui and Hoffman won the Nobel Prize of Chemistry for their independent studies which let to new theories for the interpretation chemical reactions¹⁶¹. Hoffman developed the extended Huckel method, allowing reasonable predictions of molecular conformations. Later, he won the Nobel Prize for his work showing the conservation of molecular orbital symmetry¹⁶¹. Fukui split the same prize for independent work on the Frontier molecular orbital theory.

The distribution of the electrons on a molecule, or electron density, is an important concept in the understanding of atoms, bonds and molecules reactivity^{162,163}. The

electron density is correlated with electrostatic attraction and repulsion and thus to the concept of nucleophile and electrophile¹⁶³. Electrophilic attacks take place in high electron density areas and nucleophilic attacks occur in electron deficient areas¹⁶².

The frontier molecular orbital theory (FMOT) focuses on the valence electrons and their distribution in the highest energy orbitals¹⁶². The two frontier orbitals, the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) and their interaction is essential for bond formation and breaking¹⁶². The FMOT theory states that chemical reactions should take place in the maximum overlap between the HOMO of a reactant molecule and the LUMO of another reactant molecules leading to bond formation¹⁶⁴. This overlap between orbitals leading to bond formation¹⁶⁴. This overlap between orbitals leading to bond formation can be seen increasing as the reaction path progresses. The FMOT predicts the transition states energies by approximating the slope at the reactant in a reaction path (Figure 4.1)¹⁶⁵.



Figure 4.1 Reaction pathway scheme representing how the FMOT estimate the slope to the transition states $(TS)^{165}$.

The FMOT has been widely used for the study of chemical reactions, for example diels alder reactions¹⁶⁶, metal substitutions¹⁶⁷ amongst others. In particular for dendrimeric structures this theory has been used, for example, for the study of energy flow and intense light emission of a nanostar dendrimer from the molecular orbital prespective¹⁶⁸.

The electronic effects have a determinant effect on the reactivity of molecules but their dynamic behaviour also plays a central role.

Molecular dynamics simulations allow the observation of the molecules movement with time under determined solvent, temperature and pressure conditions. There have been some molecular dynamic studies of dendrimers in an attempt to have a better understanding of these molecules behaviour, reactivity and interaction with biological targets. The effect of terminal group modification on the solution properties on amphiphilic dendrimers has been evaluated through molecular dynamic studies which revealed the formation of micelles with solvophilic monomers exposed on the surface, the global dynamics of the dendrimers can be slowed down when these solvophilic monomers are replaced with solvophobic ones, translating on an increase of the gyration radius. The capacity of forming unimolecular micelles allows these molecules to accomplish what would require many other molecules to accomplish¹⁶⁹. Other molecular dynamic studies involving glycodendrimers⁹⁹ and poly(L-lysine) dendrimers¹⁷⁰ have contributed to a better understanding of their molecular properties as flexibility and shape and how these properties can be modulated by using different monomers, different generations and different surface monomers to potentiate their use for interaction with biological target^{99, 170}.

Particularly, with recent molecular dynamics studies of PAMAM dendrimers have helped to elucidate structural features and the behaviour of these molecules. A study varying the percentage of acylation on a generation five PAMAM dendrimer revealed the overall shape is not dependent on the percentage of acyl groups on the surface. It also showed that the core and the surface are more exposed to the solvent and the high density branching units do not show much solvent penetration. Intramolecular hydrogen bonds take place but form and break and can reform¹⁷¹. Finally, it was shown that the change of solvent from water to methanol did not have an impact on these properties¹⁷¹. A different study with heterogeneously modified PAMAM dendrimers showed the distribution of the modifying groups ("caps") is dependent on the conformation of the core and branching monomers (framework), however overall shape is not altered by modifications¹⁷². A coarse grain molecular dynamic study has been done on different sizes PAMAM dendrimers conjugated with PEG500 and PEG550 and the main finding was that the conjugation with PEG does not induce aggregation but on the contrary it seems to prevent it¹⁷³. The complex formation between siRNA and either gen. 3 or 4

PAMAM dendrimers has also been recently studied using molecular dynamics simulations¹⁷⁴. The study compared the complexation energy and rate of the two dendrimer generations, the role of counter ions and the effect of the salt concentration on the complexation process¹⁷⁴.

In this PhD project, molecular dynamics simulations of PAMAM dendrimers were used to gain better understanding of their structural features and to assess the impact of glucosamine conjugation on their dynamic behaviour.

Experimental Section

Molecular dynamics simulation of saccharide modified dendrimers

The dendrimer structures were generated as described in Chapter III and saved in the pdb format. VMD¹⁵³ was utilised to convert into the mol2 file format and then imported into Maestro¹⁴⁹. Desmond was used to perform molecular dynamics simulations with explicit solvent¹⁷⁵. The dendrimer glucosamine system was built using the SPC solvation model and the size of the box was determined automatically by creating a 10 Å buffer zone around dendrimer. The molecular dynamics simulation, structure minimization and relaxation steps were performed for 4.8 ns at 300 K and 1.03 bar. Snapshot structures were recorded at every 4.8 ps.

Trajectory analysis of the molecular dynamics simulation

The trajectory from Desmond output in ".cms" format was loaded into VMD where water and ions were removed and a new trajectory was exported as a mol2 file. These files were loaded in VegaZZ 2.2.0.54¹⁷⁶ where the trajectory analysis tool was used to determined gyration radius, polar surface area (PSA), surface area and RMSD. The resulting values were imported into Microsoft Excel to be plotted. Both the distance from core to glucosamine surface and the number of hydrogen bonds where determined Maestro measuring toolkit. Accelerys Discovery using Studio 2.5 (http://accelrys.com/products/discovery-studio/) was used to generate the interpolated charge surfaces.

Electronic properties determination using Semi-empirical methods

The electronic properties of the dendrimers were determined by semi-empirical methods using Mopac 2009 $9.303W^{177}$. Input files for Mopac were created using VegaZZ 2.2.0.54 interface, choosing PM6 as an algorithm and the files were saved as ".dat" format. Notepad++ was used to edit these ".dat" files where the key words 1SFC,

MMOK, GRAPHF and VECTORS were added and the files saved as ".mop" files. Experiments were run using Mopac 2009 9.303W. Due to file size restrictions the molecules submitted for these calculations were a one quarter 'slice' of the structure for the generation 3.5 PAMAM dendrimer glucosamine. These were derived from the structures that were previously generated by molecular dynamics simulations. The first type structure that was analysed corresponded to the PAMAM dendrimer without any modifications (qdend). For the analysis of the effect of adding a single glucosamine molecule a quarter of the PAMAM dendrimer that was modified with one glucosamine was then analysed (qd1s). A quarter of the PAMAM dendrimer modified with two glucosamine molecules (qd2s) and this was used to determine the electronic properties to reflect the electronic properties of the PAMAM dendrimer modified with eight glucosamine molecules. In the same way the dendrimer modified with nine glucosamine molecules was approximated by a quarter of the PAMAM dendrimer modified with three glucosamine molecules (qd3s). In each case, six representative conformations of each dendrimer were subjected to calculations.

Results and Discussion

In Chapter III an effective method to generate 3D structures of PAMAM dendrimers was described. Here, the resulting structures were submitted to molecular dynamics simulations and 1000 structures saved as a trajectory. These were then clustered to select a significant number of representative structures to be used for glucosamine loading and distribution investigation. Additionally, the whole trajectory was analysed for a better understanding of the dynamic behaviour of these dendrimers with and without glucosamine attached.

Loading and distribution of the glucosamine molecules on the surface of a generation 3.5 PAMAM dendrimer

The generation 3.5 PAMAM dendrimer displays sixty four carboxylic end groups that are in principle available for covalent modification with glucosamine. Previous NMR studies have estimated a glucosamine loading between eight to nine glucosamine molecules per dendrimer¹, however the synthetic strategy used and properties of the molecule do not allow for a more detailed analysis using structural chemistry experiments. Therefore molecular modelling techniques were chosen to study the loading and distribution of the glucosamine molecules on the PAMAM dendrimers surface. Electronic studies were performed and interpreted according to the Frontier Orbital Theory towards a better understanding these glucosamine loadings on the dendrimer surface.

Briefly, Frontier Molecular Orbital Theory (FMOT) postulates the interaction between the HOMO (highest occupied molecular orbital) and the LUMO (lowest unoccupied molecular orbital) of molecules undergoing reaction should provide a good approximation to the reactivity¹⁶². The direction of a reaction tends to occur with increasing overlap between the HOMO and LUMO orbitals. For this study the LUMO of the glucosamine molecule was determined as well the HOMO on different patterns of glycosylated and non glycosylated dendrimers, and the difference in the energy between both determined and analysed according to this theory.

The most accurate methods to determine electronic properties of molecules make use of *ab initio* calculations and their approximations. Due to the large number of atoms in these dendrimers, the computational resources needed were beyond those available. So it was not possible to use these methods either on the whole dendrimer or on a representative portion of it. Semi empirical methods make use of the same principles as ab initio calculations, but can also utilise some experimental data. While different approximations are required, it becomes possible to study larger molecules. Even with semi-empirical methods, the dendrimers still were too large to study. Although an attempt was made to study the entire dendrimer, after 22 days of semi empirical calculations it was abandoned. Since the dendrimer has four symmetrical branches and a regular 2D structure, smaller portions of the structure were then considered as appropriate approximations for determining the HOMO-LUMO electronic properties by a semi-empirical method. As a confirmation, a small dendrimer branch with only two end groups was submitted to both ab initio (basis set) and semi-empirical calculations (PM6) (Figure 4.2). In both cases the HOMO was located on one of the terminal carboxylic acid groups. This would allow an interaction with the LUMO in the glucosamine that was located on the amine group.



Figure 4.2 The HOMO orbital from a small PAMAM dendrimer portion of a carboxylic acid end group determined by *ab initio* calculations (left) and semiempiral calculations (center). The LUMO orbital of a glucosamine molecule (right) was determined by semiempirical calculations.

Mopac software uses keywords to define calculations (including approximations) and the format of the output. In this study the following keywords were used: (i) 1SCF sets a single self consistent field calculation, no geometry optimization, (ii) MMOK allowed and increase in the rotation barrier in carbons, nitrogens, oxygens and hydrogens to make molecular mechanics corrections, (iii) VECTORS was used so that the Eigenvectors matrix could be printed in the order of their specific contributions of the atoms to the orbitals and (iv) GRAPHF allowed the visualisation of the orbitals with Jmol molecular graphics software.

These calculations of the electronic properties were applied to increasing sizes of dendrimer branches with and without glucosamine (Figure 4.3). This allowed the confirmation that the position of the HOMO was in the terminal carboxylic groups where the interaction with the LUMO of glucosamine was expected to take place. An interesting observation was that when one glucosamine molecule was present, the HOMO was located on the opposite branch to the one with the group already occupied.





Figure 4.3 Top - Increasing branches of PAMAM dendrimer modified with one glucosamine submitted to semiempirical calculations with Mopac for the determination of the HOMO energy value and location. Bottom – HOMO^d-LUMO^s gap determined with the HOMO^d energy value determined for each dendrimer structure and the LUMO^s of a glucosamine molecule (-4.61eV).

The gap between the HOMO of the dendrimer and the LUMO of the glucosamine decreases as the size of the molecules increases. According to the FMOT, the smaller the gap between the HOMO and LUMO the more likely the reaction will take place. When calculations were conducted with the larger branches there appeared to be better interactions for the reaction to occur, which would be expected since the molecular orbitals are a linear combination of atomic orbitals and the interaction with the neighbouring atoms also contributes. In the same logic, it would be expected that this gap would be even smaller if the whole dendrimer was taken into account.

Due to computational limitations for using the entire dendrimer in these calculations, a quarter of the dendrimer structure, displaying 16 terminal carboxylic groups, was selected for further analysis. Dendrimers are hyperbranched molecules and the PAMAM dendrimer structures have many rotatable bonds that allow flexibility in the structure. In an attempt to estimate the loading and distribution of the glucosamine molecules on the dendrimer surface, six representative conformations from the one quarter section of the dendrimer without glucosamine and with 1, 2 and 3 glucosamine molecules were considered in relation to LUMO of glucosamine molecule (-4.61eV). The averaged energy values of the HOMO for each dendrimer can be found in the table below (Table 4.1).

giucosamine (-4.61 ev). HOMO –dendrimer orbital, LOMO – giucosamine orbital.							
	Molecule	Number of	HOMO ^d -1	HOMO ^d	HOMO ^d -LUMO ^s		
		Glucosamines	(eV)	(eV)	GAP (eV)		
	qdend	0	-6.13	-5.97	1.36		
	qd1s	1	-7.46	-7.32	2.71		
	qd2s	2	-8.03	-7.71	3.10		
	qd3s	3	-9.00	-8.80	4.19		

Table 4.1 Average values of the HOMO energy determined for each of the six representative conformations of each dendrimer study and difference between the HOMO of the quarter of the generation 3.5 PAMAM dendrimer and the LUMO of the glucosamine (-4.61 eV). HOMO^d –dendrimer orbital; LUMO^s – glucosamine orbital.

The main observation was that the HOMO^d/LUMO^s gap is larger as more glucosamine molecules are covalently bound to the dendrimer. According to FMOT the increase in

the gap indicates that each addition of glucosamine will decrease the overlap of the HOMO and LUMO, decreasing the probability for the addition reaction to occur on this region of the dendrimer surface.

A closer look at the calculations from quarter sections of the dendrimers reveals that the energies of both HOMO and HOMO-1 with three glucosamine molecules is almost the double of the LUMO energy value for the glucosamine molecule. This makes the addition of a fourth sugar unlikely. Therefore it is possible already to establish a maximum loading of 12 glucosamine molecules per dendrimer. However, electronic effects on their own are not enough to fully understand the loading and distribution of the glucosamine molecules on the surface of these dendrimers. The observation of the positioning of the HOMO and verifying the availability of the terminal group where it is located allowed a better understanding of the glucosamine loading and distribution (Figure 4.4 and Table 4.2).



Figure 4.4 Position of the HOMO orbital on a quarter of a generation 3.5 PAMAM dendrimer with increasing number of glucosamine molecules. A – No glucosamine; B – One glucosamine; C – Two glucosamines; D – Three Glucosamines.

Both the HOMO and HOMO-1 positioning and availability were inspected for each structure. Figure 4.4 shows an example of the HOMO position for each the quarter sections of the dendrimers that were studied. The first structure is representative of all of the six conformations for the quarter of the dendrimer without any conjugated glucosamine. The HOMO/HOMO-1 orbitals are usually located in the extreme of the branches and are always available for interaction. The second structure corresponding to

one of the six conformations with one glucosamine conjugated to the surface can also be considered representative of all six conformations where the HOMO is always available. For these molecules the HOMO though not always located in the extremities of the branch is always at a considerable distance from the glucosamine. In this case, the HOMO-1 does not follow the same trend and is available in only half the structures studied (Table 4.2). The third structure in Figure 4.4 corresponds to one of the six conformations of the quarter of the dendrimer with two glucosamine molecules conjugated to the surface. This structure is also representative of what happens with these structures with the HOMO located in the interior of the branches in the middle of previously substituted end groups. This affects its availability for reaction with glucosamine. The last structure shown (Figure 4.4 D) corresponds to one of the six conformations of the quarter of the dendrimer with three glucosamine molecules conjugated to the surface. In this case, the HOMO is never available on the surface and appears on an end group in the extremity of the branch. It points inwards towards the core. Hence, it will be covered with the adjacent branch in a complete dendrimer. The availability of the HOMO and HOMO-1 for all the representative structures under study are summarised in Table 4.2.

Table 4.2 Availability of the terminal	carboxylic groups where the HOMO and HOMO
- 1 are located for each conformation	and molecule studied. + available; - not available
for the reaction.	

Number of sugars on the quarter of the gen. 3.5 PAMAM dendrimer	Availability of the HOMO	Availability of the HOMO-1	
0	+ - + + + +	+++++	
1	+ + + + -	+ - + - + -	
2	+ + +	++++	
3	+		

The addition of the first glucosamine is favourable as the carboxylic groups where the HOMOs are available. After one saccharide is covalently bound on the dendrimer's

surface, the HOMO is again located on an end group carboxylic acid making the second addition also favourable. When two sugars are already present in the quarter section of the dendrimer it becomes less likely that a third addition will take place since there is a 50:50 chance that the HOMO will be available for interaction on a carboxylic acid end group. In the last case the addition of a fourth sugar is very unlikely.

Globally, taking both electronic and steric effects into account, the addition of four to eight glucosamine molecules on the generation 3.5 PAMAM dendrimers is energetically and sterically favourable. The addition of the next set of four glucosamine molecules onto the dendrimers' surface will require overcoming a higher energy barrier and it will only find available positions for conjugation half of the time. Finally, any additionally conjugation beyond twelve glucosamine molecules in the entire dendrimer is thought to be less favoured since the energy gap is large and the carboxylic acid end group availability almost non-existent. These observations are consistent with the experimental observation of an average of 8-9 sugar molecules having been conjugated to the generation 3.5 PAMAM dendrimer surface.

Molecular dynamics simulation analysis on glycosylated PAMAM dendrimers

To understand the dynamic behaviour of these glycosylated dendrimers the first step was to simulate the dendrimer on its own for 4.8 ns. The simulation properties including the surface area, polar surface area, gyration radius and RMSD were estimated and plotted as a function of time using the VegaZZ trajectory analysis tool (Figure 4.5).



Figure 4.5 Molecular properties determined for generation 3.5 PAMAM dendrimer along a trajectory of a 4.8 ns molecular dynamics simulation. A – Gyration radius in Angstroms; B – Surface Area in square Angstroms; C – Polar Surface Area in square Angstroms; D – Root Mean Square (RMSD) in Angstroms.

The surface area graph (Figure 4.5 B) indicates there is an initial decrease on the surface area values. This is most likely result of the starting structure being generated in vacuum and use nOe constraints to stretch the initial structure. After an initial decrease the area seems to oscillate around the average value of 13965 Å². The polarity of the surface (Figure 4.5 C) seems to follow similar pattern along the trajectory.

In bioinformatics, root mean square deviation (RMSD) is used to measure distances between atoms after structure superimposing. This measure is usually applied to proteins to assess flexibility and folding¹⁷⁸⁻¹⁸⁰. However, it has also been applied to different systems as nucleic acids¹⁸¹. The rigid body RMSD formula for two sets of n points a and b is:

$$RMSD_{(a,b)} = \sqrt{\frac{1}{n} \sum_{i=1}^{n} ||a_i - b_i||^2}$$

The units for RMSD are usually angstroms^{179, 180}.

The RMSD analysis of the PAMAM dendrimer shows a plateau after the first 1400 ps (Figure 4.4d). This means the molecule explored the conformational space with similar differences when compared to a reference structure. However, further changes in conformation space were explored where an increase of the RMSD values towards the end of simulation were observed. Although the RMSD reached a plateau after 1.4 ns, RMSD values of approximately 11 Å indicate flexible structure. This plateau is consistent with the profile that was obtained for the gyration radius where after the 1400 ps only limited fluctuations occurred. These fluctuations indicated that the shape of the structure did not undergo major changes during the simulation period.

Closer inspection of the overlay of representative structures also indicate that the core and inner branching units are fixed and that most of RMSD changes likely result from the movement of the outer sphere of the dendrimer (Figure 4.6). The three superpositioned structures in Figure 4.5 correspond to a quarter of the dendrimer at three different time points. With their core overlapped, it is possible to see that the first branching generation in the dendrimer practically does not change during the simulation. From the second generation onwards toward the end groups, the structures start to show changes with the greatest changes observed in the last 1.5 generations of the dendrimer, this being consistent with the described above.



Figure 4.6 Superimposition of a quarter of a generation 3.5 PAMAM dendrimer at different time points of the trajectory of a 4.8 ns molecular dynamics simulation. Blue – Structure after 1.4 ns of the simulation; Dark pink - Structure after 3 ns of the simulation; Red - Structure after 4.2 ns of the simulation. Black – Dendrimer core, superposition point.

The second step in the molecular dynamic simulation was to then add one glucosamine to the whole dendrimer surface and then inspect the differences in the dynamic behavior. Experimentally it is known that at the end of reaction the excess reagents (i.e. glucosamine and EDC) and co-products (i.e. urea) remain in solution until they after are dialyzed from the solution during workup. To evaluate the possible effect of the presence of these components of reaction mixture on the dendrimer conformation, molecular dynamics simulations of the dendrimer modified with one glucosamine molecule were performed in presence and absence of EDC, urea and glucosamine molecules. The molecules of reagents and co-products were randomly positioned between the dendrimer branches and around dendrimer surface. The dendrimer surface area values were higher at the beginning of the simulation, however towards the end of the simulation both profiles converged to the same value (Figure 4.7). The surface of the dendrimer is higher in the presence of the reagents, which is most likely due to the presence of EDC positioned between branches, resulting in the end groups are more

exposed. Experimentally it is necessary that the EDCI first undergo reaction with the carboxylic acid groups. It was also observed by NMR that the EDCI derived urea is associated with the dendrimer glucosamine product and can only be effectively removed by dialysis (NMR spectra in Chapter II).



Figure 4.7 Molecular properties determined for generation 3.5 PAMAM dendrimer modified with one glucosamine molecule along a trajectory of a 4.8 ns molecular dynamics simulation. Orange – simulation performed in the absence of reagents; Dark Pink - simulation performed in the presence of reagents; A – Gyration radius in Angstroms; B – Surface Area in square Angstroms; C – Polar Surface Area in square Angstroms; D – Root Mean Square (RMSD) in Angstroms.

This trend is also observed for the gyration radius (Figure 4.7). The Gyration radius can be used as measure of compactness of a structure¹⁸². It measures the mean deviation of the monomers of a structure in relation to its mass center¹⁸².

In the presence of the reagents and side products, the structure is moving more from its centre of mass, allowing the end groups to be available to interact with the reagents. However, the two dendrimers structures converge towards the end of the simulation due to ECD molecules diffusing from the interior of the dendrimer. This trend leading to convergence is observed in all these simulated properties after approximately 3 ns and for that reason reagents were not taken into account for further calculations.

In first part of this Chapter it was shown that the loading of the glucosamine molecules would most likely be limited to a dendrimer with eight glucosamine molecules being covalently bound to the surface carboxylic acid moieties. However, other loadings are possible and cannot be ignored. In an attempt to analyse the dynamic behavior of what would be considered a biologically active dendrimer, molecular dynamics simulations were performed with three different glucosamine loadings on the generation 3.5 PAMAM dendrimers. These were modified with seven, eight and nine glucosamine molecules. Their molecular properties were determined and compared (Figure 4.8).



Figure 4.8 Molecular properties determined for generation 3.5 PAMAM dendrimer modified with variable number of glucosamine molecules along a trajectory of a 4.8 ns molecular dynamics simulation. Brown – dendrimer modified with seven glucosamine molecules; Green – dendrimer modified with eight glucosamine molecules; Blue – dendrimer modified with nine glucosamine molecules; A – Gyration radius in Angstroms; B – Surface Area in square Angstroms; C – Polar Surface Area in square Angstroms; D – Root Mean Square (RMSD) in Angstroms.

The three dendrimers under study displayed very similar gyration radius values. Throughout the simulation the dendrimer with nine glucosamine molecules appeared to be the most compact. For the surface area values the three conjugates displayed profiles with similar values, however the dendrimer with nine glucosamine molecules was shown for a significant period of time lower values than the one with eight sugars. This was consistent with the 9-glucosamine dendrimer having a more compact structure, which was not expected since this molecule has the highest number of atoms. The surface polarity of these dendrimers is proportional to the amount of glucosamine that is conjugated. Finaly for the RMSD there was a clear difference in the values obtained for the dendrimer modified with eight glucosamines. This suggested that this molecule was exploring larger conformational space. However with longer simulation times, these properties reached a plateu and converged. The observed difference in behaviour of the dendrimer with eight glucosamines on the surface and the other two dendrimer glucosamine conjugates is interesting since this is the only molecule with a even distribution of saccharide molecules on its surface. It appears to be the most flexible and might be an indication that less evenly distributed glucosamine modifications may result in a more rigid dendrimer that has a smaller conformational space..

Despite the small variations in the molecular properties of these dendrimers, after 4 ns, they seem to stabilise and converge. Hence, for these properties, a single dendrimer glucosamine could be used as a representative structure for these three molecules with different loadings (analogues). In this case, the dendrimer chosen for future studies was the generation 3.5 PAMAM dendrimer that was conjugated with eight glucosamine molecules. Based on the computational HOMO-LUMO reactivity studies and the observations made during conjugate synthesis about loading, this molecule was thought to be a good structure to use.

To try to assess the impact of saccharide addition on to the surface properties of the dendrimers and to inspect differences between biologically active (gen. 3.5 PAMAM dendrimer modified with eight glucosamine molecules) and inactive (gen. 3.5 PAMAM dendrimer without modifications and the gen. 3.5 PAMAM dendrimer modified with one glucosamine molecule) dendrimers, a comparison between the properties of the dendrimer without modification, dendrimer with one glucosamine and dendrimer with eight glucosamine with one glucosamine with one glucosamine without modification.



Figure 4.9 Gyration radius values determined for generation 3.5 PAMAM dendrimer modified with variable number of glucosamine molecules along a trajectory of a 4.8 ns molecular dynamics simulation. Blue – dendrimer without glucosamine molecules; Orange - dendrimer modified with one glucosamine molecule; Green – dendrimer modified with eight glucosamine molecules.

Looking at the gyration radius it is possible to say that the more sugars present conjugated to the dendrimer the less compact the structure. However at some time points the dendrimer with one glucosamine molecule on its surface seemed more compact than the dendrimer without modifications. Another observation is that the three structures seem to converge towards the end of the modelling trajectory (Figure 4.9). A longer simulation is necessary to confirm if this convergence is real since there were other points of the trajectory where the three values are the same but diverge a few picosecond after (for example around 3 ns). Due to computational resource and the purposes of this work, longer simulation periods were deemed unnecessary since the flexibility of dendrimer molecules remains, even when a plateu would be reached.



Figure 4.10 Surface Area (top) and Polar surface area (bottom) values determined for generation 3.5 PAMAM dendrimer modified with variable number of glucosamine molecules along a trajectory of a 4.8 ns molecular dynamics simulation. Blue – dendrimer without glucosamine molecules; Orange - dendrimer modified with one glucosamine molecule; Green – dendrimer modified with eight glucosamine molecules.

The profiles found for surface area and polar surface areas for these molecules are very similar for each molecule (Figure 4.10). This would be expected since there are more atoms on the conjugate surface which should mean a higher surface area. Glucosamine

is a polar, polyhydroxylated molecule, so it is plausible that its presence on the surface would result in a higher polarity than observed for the precursor dendrimer. However, knowing these are flexible structures if the branches bearing the glucosamine tended to fold towards the core there would be no increase on the polarity of the surface. This is actually observed at some points of the trajectory where the polarity and the area of the surface of the dendrimer with one glucosamine is the same of inferior to the values of the dendrimer without any glucosamine.



Figure 4.11 RMSD values determined for generation 3.5 PAMAM dendrimer modified with variable number of glucosamine molecules along a trajectory of a 4.8 ns molecular dynamics simulation. Blue – dendrimer without glucosamine molecules; Orange - dendrimer modified with one glucosamine molecule; Green – dendrimer modified with eight glucosamine molecules.

The RMSD values are a measure of flexibility as it indicates the average movements of the atoms during the simulation. Plateaus usually indicate a vibrational state with a molecule being kept in the same conformation, in relation to the starting conformation. However this may not be true for PAMAM dendrimers as shown previously. The dendrimer without glucosamine on its surface seems to reach a plateau before 2 ns in the simulation (Figure 4.11 blue series). The units on the outer surface continued to change conformation almost until to the end of the trajectory when it starts to explore completely different conformational space. The dendrimer with one glucosamine on its

surface seems to be in constant motion; however, there are no jumps to very different conformations as it oscillates throughout most of the trajectory (Figure 4.11 orange series).

Finally, the dendrimer with eight glucosamines on the surface presents several plateaus in its profile (before 2 ns; 2.5 ns and after 4ns). This means it passes through a few different conformations. With time this dendrimer glucosamine appear to explore an increasingly larger conformational space (Figure 4.11 green series).

Considering the profiles of these molecules, the more conjugated glucosamine the more flexible and larger conformational space of the dendrimer structure is explored.

The number of hydrogen bonds for each the dendrimers was investigated because Hbonds between the branches would be expected to influence the flexibility of the dendrimer. It may also be possible that the availability for interactions with the biological target could be influenced by intramolecular H-bonds. Possible hydrogen bond formation between branching units, defined as DBB on chapter III, highly influences the flexibility of these molecules and therefore it is not expected based on the previously determined properties.

In general, hydrogen bond lengths can vary between 2 to 3.5 Å, being stronger the shorter the distance. The angle between the hydrogen bond donor and acceptor is also an important factor. The bond is stronger if the two are aligned. In this study, the criteria used for possible hydrogen bond formation was a maximum distance between hydrogen bond donors and acceptors of 2.5 Å, a minimum donor angle of 120° and a minimum acceptor angle of 90°.



▲ Dend □ D1S ● D8s

Figure 4.12 Number of hydrogen bonds between branching units (DBB-DBB) determined for generation 3.5 PAMAM dendrimer modified with variable number of glucosamine molecules along a trajectory of a 4.8 ns molecular dynamics simulation. Blue – dendrimer without glucosamine molecules; Orange - dendrimer modified with one glucosamine molecule; Green – dendrimer modified with eight glucosamine molecules.

This figure 4.12 shows that a maximum of three hydrogen bonds occur in the two conformations adopted by the dendrimer without glucosamine. One H-bond is adopted by the dendrimer modified with one glucosamine. These values are low in comparison to the number of hydrogen bond donors and acceptors within the dendrimer structure. In general, it can be considered the profiles are consistent with the observation of highly flexible molecules and that the addition of sugar in the surface does not affects the dendrimers flexibility.

The presence of intramolecular hydrogen bonds between the branching units (DBB) and the surface units (DBC) could also have an impact on the structure flexibility, further more these would contribute to compact structures and restrict the possible interactions between the dendrimer surface and the biological target.



◆ Dend □ D1S ● D8S

Figure 4.13 Number of hydrogen bonds between branching units and surface units (DBB-DBC) determined for generation 3.5 PAMAM dendrimer modified with variable number of glucosamine molecules along a trajectory of a 4.8 ns molecular dynamics simulation. Blue – dendrimer without glucosamine molecules; Orange - dendrimer modified with one glucosamine molecule; Green – dendrimer modified with eight glucosamine molecules.

The number of hydrogen bonds found is considerably higher than between branching units, however this does not mean the possible interactions with the biological target would be compromised, A maximum of 38 hydrogen bonds are observed out of a maximum of 64 end groups each bearing two negatively charged oxygen atoms, which reduce this high value to only a quarter of the total possible interactions (Figure 4.13).

It is possible, and consistent with the surface area and gyration radius observations, that these hydrogen bonds are contributing to the more compact structures of the dendrimer without and with one glucosamine, as they seem to happen in higher number for these two molecules.

Finally, the number of hydrogen bonds between the glucosamine substituent (DBD) and the branching units (DBB) was estimated. These values may be particularly important for the availability of the saccharide for interaction with the biological target.



Figure 4.14 Number of hydrogen bonds between branching units and glucosamine units (DBB-DBD) determined for generation 3.5 PAMAM dendrimer modified with variable number of glucosamine molecules along a trajectory of a 4.8 ns molecular dynamics simulation. Orange - dendrimer modified with one glucosamine molecule; Green – dendrimer modified with eight glucosamine molecules.

The dendrimer with one glucosamine presents a maximum of 1 hydrogen bond between the glucosamine and the branching units and this situation is only present in 6% of the conformations studied and being therefore not representative of the whole trajectory. The dendrimer with eight glucosamine is considered the biological active dendrimer. It forms a higher number of hydrogen bonds with 26% of the conformations presenting one hydrogen bond with branching units, 8% present two hydrogen bonds, 1,7% three and only one structure presents four hydrogen bonds with branching units (Figure 4.14). These results raised some concern because if the glucosamine moieties were involved in hydrogen bonds with lower generations of the branching units, this might mean that they were folding towards the centre of the molecule. Such inward folding would suggest there was a diminished capacity of the glucosamine moieties to be available for intermolecular interactions. However, if these bonds were taking place with higher generations the glucosamine molecules there would still be available for interaction with the biological target. In order to assess the real impact of the occurrence of these intra molecular hydrogen bonds, visual inspection of the structures presenting the higher number of bonds was performed (Figure 4.15).



Figure 4.15 Structures presenting higher number of intra molecular hydrogen bonds between saccharide units and branching units. The branching units are represented as the molecular surface; the terminal units are represented as tube and the saccharide units as CPK. Only polar hydrogen atoms are displayed. A – generation 3.5 PAMAM dendrimer modified with one glucosamine; B - generation 3.5 PAMAM dendrimer modified with one glucosamine. The green arrow points to the glucosamine unit buried on the molecular surface representing the branching units and thus less available for interactions with the biological target.

The dendrimer with one glucosamine molecule on its surface had one hydrogen bond between the glucosamine unit and the branching unit (Figure 4.15A) at the surface. This appeared not to have any influence on the availability of the saccharide nor contributing to a formation of a compact structure. The dendrimer displaying eight glucosamines on its surface displayed four hydrogen bonds between the glucosamine unit and the branching unit (Figure 4.15B). This did not present a worrying situation since these bonds did not affect the availability of the glucosamines to interact with the biological target. The glucosamine appeared to be fully available on the surface of the dendrimer. One exception was observed and is shown in Figure 4.15 B with a green arrow. In this case the glucosamine molecules seem partially buried within and in between the branches. The end group was therefore less available but not completely inaccessible. Furthermore, the image (Figure 4.15) displays "the worst case scenario" for each type of molecule, where the higher number of intramolecular hydrogen bonds occurs, which happens in snapshot conformations and thus cannot be considered as representative behavior.

Charge and its distribution in the dendrimers are thought to be important for their interaction with the biological target. Here, it is hypothesized that the biological relevance of these dendrimers is due to their interaction with the LPS recognition system, particularly with the accessory protein MD-2. This is the protein where the LPS bind¹⁸³. Detailed studies of these interactions will be reported on Chapter V, however, when trying to understand the difference between inactive and active PAMAM dendrimers it becomes important to assess this property too. Interpolated charge surface were generated for the generation 3.5 PAMAM dendrimer (inactive) and for the generation 3.5 PAMAM dendrimer with optimal loading of eight glucosamine molecules on the surface.



Figure 4.16 Interpolated charge surface for: A - a generation 3.5 PAMAM dendrimer and B - a generation 3.5 PAMAM dendrimer modified with eight glucosamine molecules on its surface, the red arrows point out three of these glucosamine molecules. The red areas correspond to negatively charged residues and blue to positively charged residues.

The surfaces that have been studied do reveal a positively charge region through the branching and toward the core of both dendrimers where the protonated amine groups are located (Figure 4.16). The negatively charged areas are found towards the surface where the deprotonated carboxylic groups are. The main differences that are observed are precisely on the surface. The presence of the glucosamine molecules reduces the negative charge on the surface (Figure 4.16 B). This allows for a more relaxed structure with higher surface area and a more balanced charge distribution, possibly contributing for the interaction with the biological target.

Conclusion

The electronic studies revealed a range of possible loadings and distributions, consistent with the heterogeneity found in the experimental samples. This range is determined not only by pure electronic effects but also by the different conformations adopted by these molecules. It was possible to establish a range from 8 to 12 glucosamine molecules, which comprises the experimental estimates of 8-9 glucosamine molecules per dendrimer.

During synthesis the glycosylation of the dendrimer occurs in the presence of excess reagents, and these are known to remain in solution in trace levels, being speculated that they can be trapped within the dendrimers branches. This study revealed the presence of excess reagents in solution does not have an impact on the dynamic behavior of dendrimers and can for that reason be disregarded. This is an important factor as the presence of reagents in solutions increases the size of the system and therefore the computational time required.

When assessing the dynamic behavior of known active PAMAM dendrimers, the range of sugar loading determined above was taken into account. Since the most favorable structure would be the dendrimer with eight evenly distributed glucosamine molecules, this loading plus/minus one was considered. The three molecules were simulated and their molecular properties estimated. This study revealed that the dynamic behavior of these molecules followed the same profile, converging in most cases. The exception was the RMSD where the dendrimer with eight glucosamines shown to explore larger conformational space. For these reasons the generation 3.5 PAMAM dendrimer was considered representative of the active dendrimer population and was used for comparison with inactive PAMAM dendrimers and will be used for interaction studies with the hypothesized biological target.

From the dynamic behavior of generation 3.5 PAMAM dendrimers modified with variable number of glucosamine it was possible to identify flexibility as an important property of these molecules. Flexibility seems to be a major contributor to the loading

and distribution of the glucosamine molecules on the surface and also having a role in the surface properties of the dendrimers.

The major differences found between the active and the inactive PAMAM dendrimers which might have an impact on their activity were, larger conformational space explored and thus higher flexibility (RMSD measurements) and slightly less compact structure (gyration radius). The surface area and the polarity of the dendrimers' surface are believed to be determinant for their biological role. The presence of intramolecular hydrogen bonds showed to not have an impact on availability of glucosamine for intermolecular interactions.

Chapter V – Docking Studies with the LPS Recognition System

Introduction

Efforts to study the conformation and dynamic behaviour of glycosylated generation 3.5 PAMAM dendrimers are described in previous chapters. These efforts have resulted in greater insight, so that it is now possible to examine computationally the mechanism of the biological activity observed for these dendrimers.

The glycosylated PAMAM dendrimers inhibit cytokine release after primary cells are exposed to LPS. The LPS triggered inflammatory response has two crucial steps. First the LPS is thought to bind to the MD-2 protein and this is followed by the dimerisation of the TLR4-MD-2 complex^{63, 184} (Figure 5.1). The glycosylated generation 3.5 PAMAM dendrimers appear able to affect this biological pathway¹ to inhibit the cytokine response. The mechanism by which these molecules prevent the inflammatory response is not known. In this project, it is hypothesised that the biological relevance of the glycosylated dendrimers is due to their interaction with MD-2 protein which in turn prevents binding of the LPS. The aforementioned studies on the conformation and dynamic behaviour of glycosylated dendrimers suggests that it might be possible to computationally examine the possible interactions of these dendrimers with their potential biological target. The interaction studies between the glycosylated dendrimers and the MD-2 protein using molecular modelling techniques were conducted.

The LPS recognition system involves four different proteins that exist extracellularly. The LPS binding protein (LPB) binds an LPS molecule from LPS aggregates making it available to the transport protein CD14. CD14 then positions LPS so that it is transported to MD-2 which is followed by a complex interacting with the membrane bound TLR4 receptor. This then initiates a cascade of intracellular events that culminate in inflammatory cytokine and chemokine release⁶³ (Figure 5.1).



Figure 5.1 Extracellular events of the LPS recognition by pattern recognition receptors, adapted from Jerala, 2007. A and B represent the different possibilities regarding the order of events. A – CD14 delivers the LPS to MD-2 bond to TLR4; B – LPS binds to MD-2 prior to MD-2 bind to TLR4.

The LPS binding protein (LBP) is a glycoprotein and is a member of the lipid transfer/LPB family and it is composed of two domains¹⁸⁵ as confirmed by a model for LBP based on the recent BIP crystal structure¹⁸⁶. This family includes the bactericidal/permeability-increasing protein (BIP). Each of the two domains of LBP is composed of an antiparallel β -strand layer that twists around a long α -helix. The N-terminal of LPB contains cationic amino acid residues thought to be involved in the LPS binding¹⁸⁷. The C-terminal region is thought to be involved in the transfer of LPS from the LBP to CD14. This region of the LBP may also be involved in interaction with membrane¹⁸⁸.
Like LBP, CD14 is also a glycoprotein. CD14 can be found in the serum in a soluble form. The protein is also found anchored in the cell membrane⁸. CD14 is a phospholipid transport protein and is composed of 11 leucine rich repeats. Its structure has been recently solved as a dimer which resembles the 'horse shoe' fold that is typical for TLR receptors^{189, 190} (Figure 5.2).



Figure 5.2 Ribbons representation based on the crystal structure of two monomers of the glycoprotein CD14 $(1WWL)^{189}$. Blue – N-terminal; Red – C-terminal; the arrow points to putative LPS binding site.

The N-terminal region forms a hydrophobic pocket thought to be involved in binding with LPS because its dimensions allow for stabilization of the LPS acyl chains^{191, 192}. Other residues like Glu7–Asp10, Asp9–Phe13, or Leu91–Glu101 in human CD14, close to the putative binding pocket have been identified as involved in signalling events¹⁸⁹. ¹⁹²⁻¹⁹⁴. Though these residues are not directly involved in the LPS binding they constitute potential interaction sites for other proteins involved¹⁸⁹. CD14 is also believed to bind the carbohydrate chains of LPS. This binding appears to occur on the upper face grooves of CD14 making it difficult to assess the real importance of the hydrophobic pocket for the signalling pathway^{195, 196}. The mechanism of LPS delivery to the LPS to MD-2 monomer by CD14 remains to be explained.

The last protein involved in LPS recognition before complex formation with the cell receptor is MD-2. This protein has only been identified along with its structure and function in the last few years.

MD-2 structure consists of two β sheets, one with three antiparallel β strands and the other with six¹⁹⁷. Between the sheets, a deep hydrophobic cavity was found where the lipidic chains of lipid IVa were bound. Overall, MD-2 structure showed high positive charges in the entrance of the cavity and a highly hydrophobic interior¹⁹⁷. The crystal structure of human MD-2 was determined together with the LPS antagonist Lipid IVa¹⁹⁷ (Figure 5.3).The resolution of this structure has shown MD-2 as the central point of LPS recognition and defining hydrophobic interactions as the driving force for the interaction between MD-2 and LPS.



Figure 5.3 Crystal structure of the human MD-2 protein¹⁹⁷ (2E56). Blue – protein backbone displayed as cartoon; Grey – surface of the hydrophobic pocket where the LPS binds.

In early 1999, a review from Wright first hypothesised the existence of a factor or an adaptor protein for LPS binding when involved in a TLR4 mediated response¹⁹⁸. In the same year, Shimazu et al reported a new protein which they called MD-2. This new

protein shares 23 % homology with the previously isolated MD-1¹⁹⁹. This MD-1 protein had been shown to be physically bound to RP105, which is a protein expressed on the surface of lymphocytes and similar to TLR4. The relation of MD-2 and TLR4 was then investigated by the same authors who verified that cells expressing TLR4 were also positive for MD-2. Confocal microscopy revealed that the two proteins were co localised and immuneprecipitation assays confirmed that these two proteins were interacting with each other¹⁹⁹.

Further research showed the importance of the association of MD-2 with TLR4 for LPS recognition^{200, 201}, however the binding site of LPS remained unknown until Viriyakosol et al reported that MD-2 is responsible for LPS binding. They showed this binding occurs with a 1:1 molar ratio²⁰². Six out of the seven cysteine residues of MD-2 have been identified as critical for its biological function²⁰³⁻²⁰⁵. A peptide sequence similar to those of endotoxin-neutralising peptides was found to bind to LPS and be relevant for its signalling²⁰⁶⁻²⁰⁸. Different studies with MD-2 from various organisms and with compounds with similar structure to lipid A have revealed the main recognition motifs on MD-2 are within the hydrophobic portion and the arrangement of the negative charges on MD-2^{63, 209-211}. These studies did not find the disaccharide portion of LPS relevant. Species specificity for ligand recognition by MD-2 has also been shown^{63, 201}.

More recently, a model for a TLR4-MD-2 complex recognition of LPS has been proposed based on the crystal structure of the extra cellular TLR4-MD-2-LPS complex¹⁸⁴. Based on this model it was hypothesised that the binding of LPS induces the dimerisation of the TLR4-MD-2 to form an "m" shaped symmetrical multimer. However, this model is a static snapshot and does not provide information of the order of the events nor does it show how LPS may induce dimerisation of the TLR4 with MD-2. This in turn leads to the dimerisation of the intracellular domains of TLR4 receptor, triggering the cascade reaction which culminates with the release of pro-inflammatory chemokines and cytokines¹⁸⁴. The crystal structure revealed that LPS interacts both with MD-2 and TLR4, furthermore, it was shown that both the lipid and saccharide portions of LPS are involved in the interactions¹⁸⁴ (Figure 5.4).



Figure 5.4 Crystal structure of the human TLR4-MD-2-LPS complex after dimerisation $(3FXI)^{184}$. The TLR4 and MD-2 monomers are represented as ribbons (colours red and blue MD-2, cyan and brown are extracellular domains of TLR4) and the LPS molecules as sticks. Orientation – C-terminals of capped TLR4 (missing transmembrane and intracellular domains) would be interacting with the membrane as indicated by arrows. The saccharides needed for crystallisation were removed for clarity.

The importance of the saccharide portions of LPS and of the ionic interactions has been recently reinforced in a study by Meng et al. that also emphasis the species specificity of the LPS recognition system^{212, 213}. Human and mouse TLR4-MD-2 complexes react differently upon lipid IVa binding. In humans lipid IVa acts as an antagonist of LPS but in mice it acts as agonist. This had already been shown by different authors^{63, 201}, however, these differences were used to elucidate the role of the saccharide portion of the lipid IVa on binding and complex dimerisation²¹³. An inversion in the charge in the mouse TLR4 residues located at the dimerisation interface results in the absence of response to lipid IVa. In the same way, the charge inversion on the human TLR4 residues resulted in a gain of response towards the lipid IVa molecules. These results show not only that there is species selectivity of this LPS recognition system but also that there are important ionic interactions between the phosphorilated saccharides of lipid IVa and LPS²¹³.

Much progress in the understanding of LPS recognition and the consequent activation of the inflammatory response has occurred in the last 2 years. Nevertheless, some steps remain unclear. Interestingly, there is no clear information about whether LPS binds to MD-2 or to the TLR4-MD-2 complex. A thorough search of the available literature indicates that there are two reports where this issue is discussed. Akashi *et. al* reported the Kd of complex of lipid A-MD-2-TLR4 to be ten to twenty times lower²¹⁴ than the Kd values reported previously for both lipid A-MD-2 or lipid A-CD14²⁰². However, nowhere in this report do the authors show that the complex between TLR4-MD-2 is formed prior to the binding lipid A to MD-2. Furthermore, in 2006, Visintin et al, suggest that the only stable complexes are those with the three components, LPS, MD-2 and TLR4, although the last two can interact "promiscuously"²¹⁵. This associated with the fact that no crystal structure has been reported of the TLR4-MD-2 without lipid A or other agonist of the system, suggests that LPS bind primarily to MD-2 in the blood stream and only then the complex with TLR4 is formed. This is a key assumption when defining the components of the LPS recognition system to include as targets in the interaction studies.

Molecular docking methods were then used to further investigate the interactions between the glycosylated dendrimer and the LPS recognition system.

Molecular docking is a methodology which allows the study of the interactions between different molecules with many software packages available for this type of study. Knowledge of the molecular system is crucial for the decision about what type of software package to use. In general, it is possible to classify the software packages based on the molecular system that they are generally applied to. Often they can be either directed towards protein-protein (protein-DNA) interactions or small molecule-protein interactions. All docking software can perform either (i) rigid docking, where the conformation of the molecules it is not altered during the docking process or (ii) flexible docking, where the conformation of part or whole molecules change during the best fit search.

Dendrimers are hyperbranched polymeric structures of large molecular dimensions, their size and the absence of atom type parameters in docking software were major limiting factors in the study of their interaction with LPS recognition system.

Two key determinant aspects to examine molecular interactions are molecular shape and electrostatics. The dendrimeric nature of the system does not allow for their treatment as proteins or small ligand. Therefore as a strategy several software packages were assessed to determine if it might be possible to evaluate the different interactions of glycosylated PAMAM dendrimers with the LPS recognition system These studies had to be conducted separately and then compared and combined in an attempt to gain insight about how the glucosamine PAMAM dendrimers may have inhibited the release of cytokines.

Experimental Section

Crystal structures

The structures of proteins used for docking studies were obtained through the RCBS website (www.rcsb.org). The systems to be studied were the crystal structures of the mouse TLR4-MD-2 in a complex with eritoran (2z64), the human MD-2 complexed with lipid IVA (2e59) and the complex of human TLR4-MD-2 (3FXI).

Docking studies with Hex 5.0

The docking studies carried out with Hex involved the use of two different protocols. The first was a shape based protocol and the other was a protocol that included both shape and electrostatics. All other parameters were the same for both protocols. The scan steps were set to 0.75 with 2 substeps. The order of the docking correlation was set to 25 for the steric scan and 25 for the fine search. The grid size was set to 1Å. A total of 100 solutions were saved. Of these the representative structures of the first 20 clusters were saved as pdb files for further analysis. For validation both protocols were used to dock MD-2 with TLR4 as in the crystal structure (2Z64). The studies of the gen. 3.5 PAMAM dendrimer and the generation 3.5 glycosylated PAMAM dendrimer against the MD-2 and TLR4-MD-2 complex were performed with the shape and electrostatics protocol only. Twenty different conformations of each biologically active and inactive dendrimer were used as ligands. This gave in total 800 solutions (resulting complexes) in this study.

Docking studies with Patchdock

Patchdock was used with all default parameters, except that the maximum surface overlap had to be changed to a lower value of -2. The default value gave non-realistic structures with dendrimer branches entering the protein core. The 20 lowest energy structures were saved as pdb files for further studies. The complete parameter file for Patchdock can be found in Appendix 5.1. The same protocol was used to dock MD-2

with TLR4 for validation to compare with the crystal structure (2Z64). Once validated, the protocol was used to study the possible interactions of the generation 3.5 PAMAM dendrimer and the generation 3.5 glycosylated PAMAM dendrimer with both the MD-2 and TLR4-MD-2 complex.

Docking studies with GRID22 software

Three components of the GRID (Molecular Discovery Ltd) software package were used for these studies. Greater (Molecular Discovery Ltd) was used to generate the ".kout" files for all of the molecules which were studied. Glue (Molecular Discovery Ltd) was used for docking. This requires either ".kout" or "mol2" files of ligands used in the docking process. So "pdb" files were converted to .kout. For the docking process, all available probes were selected to generate molecular interactions fields (MIFs). A maximum number of 100 binding sites were used with an energy cut off of -100 Kcal/mol. The maximum iterations value was set to 120. For ligand flexibility 5 rotatable bonds were allowed and the electrostatic term was included for interaction energies calculation.

For validation of this software Lipid IV A was docked against MD-2 as in the crystal structure (2E59). The other studies performed with Glue were, glucosamine and three small dendrimer branches of increasing sizes as ligands were docked against the whole MD-2 and glucosamine was docked onto the human TLR4-MD-2 complex (3FXI). For these last experiments, due to the large size of the complex, the target was divided into several overlapping target volumes defined as a box with a side of 30 Å and the glucosamine was docked into each target volume separately.

Data analysis of the docking solutions

For the visualisation of the outputs from the docking studies and to generate images, Discovery Studio Visualise (Accelerys) was used. This software was also used in the processing of the docking solutions to identify atoms from ligand and protein that are less than 4 Å apart from each other. Furthermore, the rebol script, designed to utilize the molecular graphics capabilities of Vega ZZ, has been used to automate processing the 800 solutions of the docking of the two dendrimers with the MD-2 protein. This script reported every atom from the dendrimer at a distance smaller than 3 Å from an atom in the protein. The complete script can be found in Appendix 5.2. The resulting files were then exported to Microsoft Excel and then the graphs were plotted. For energy scaling of the interactions, the number of interactions from each solution was multiplied by their energy value. The energy calculations were only performed for Hex solutions.

Molecular dynamics simulation of the docking solutions

The "pdb" file with the docking solution from Hex was loaded into Maestro and Desmond was used to perform molecular dynamics simulation with explicit solvent. The system was built using the SPC solvation model and the size of the box was determined automatically by creating a 10Å buffer around the glycosylated dendrimer and the MD-2 protein. The molecular dynamics simulation was performed for 4.8 ns at 300K and 1.03 bar. This included structure minimisation and relaxation steps. Snapshot structures were recorded at every 4.8 ps.

The contact surface areas between the glycosylated dendrimer and MD-2 from selected frames of the 4.8 ns trajectory were determined using Chimera software package (www.cgl.ucsf.edu/chimera), setting the cut off for van der Waals interactions to 4 Å.

Results and Discussion

The first step was to screen the available software packages to determine which software would be able to cope with the dendrimer structure interacting with a protein. In summary, the packages that are dedicated to small molecule-protein interactions could not cope with the number of atoms in the dendrimer. Most of the packages dedicated to protein-protein interactions were built to recognise structures based on the amino acid residue names and so were not capable of recognising the dendrimer. However, two protein-protein interactions software packages (Hex and Patchdock) did have the capacity to represent the correct dendrimer structure. Due to the large size of the dendrimer and software limitations, flexible docking was not possible, thus rigid docking was performed on twenty different representative conformations of the dendrimers that were studied (Figure 5.5). Both Patchdock and Hex software packages were used to evaluate shape influence on the interactions. GRID protocol was used to determine the electrostatic contributions for the interactions. The ligand molecules included in the study were a generation 3.5 PAMAM dendrimer and a generation 3.5 PAMAM dendrimer with eight glucosamine molecules on its surface. The target molecules included in the study were the accessory protein MD-2 and the TLR4-MD-2 (Figure 5.5). It was not possible to use the whole dendrimer molecule to adequately assess the electrostatic contribution to the interactions, consequently smaller portions and individual glucosamine molecules were used as ligands for docking using GRID and GLUE software.

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SHAPE

- PatchDock, HEX
- 20 Conformations of each ligand
- 20 Solutions for each conformation
- Assessment of the dynamic behaviour of interaction

ELECTROSTATICS

- Use branches of dendrimer and glucosamine
- GRID
- 20 Solutions for each branch conformation
- Build the dendrimer from the branch
- Assessment of the dynamic behaviour of interaction



Figure 5.5 Strategy adopted for the study of the interactions between the PAMAM dendrimers and MD-2 protein. The text in grey, represents the steps of the strategy that could not be performed due to the results obtained in the first step.

Validation of Patchdock, Hex and Glue software packages

Hex and Patchdock are two different software packages developed for the study of protein-protein interactions. Their algorithms and scoring functions are different though. Patchdock reads each structure and transforms them into Conolly surface representations^{216, 217} that include flat, concave and convex patches²¹⁸. The complementarities between patches are assessed and then given a scoring function, which takes into account the shape fitting and desolvation energy (i.e. energy required to break molecule-solvent interactions). The results are clustered based on the RMSD values of each transformation²¹⁸.

The crystal structure of the mouse TLR4-MD-2 complex (2z64) was used to evaluate if the software could reproduce the protein complex structure. The mouse TLR4-MD-2 complex was used, since at the time of these studies there was no crystal structure of the human cell receptor complex.



Figure 5.6 Results obtained for the interaction study of mTLR4 extracellular domain and mMD-2, using Patchdock and target was A –TLR4 pdb file without any of the saccharides co-crystallised and present in the original pdb file (2Z64); B - TLR4 pdb file with all of the saccharides co-crystallised and present in the original pdb file (2Z64); C -TLR4 pdb file only with the saccharides, inside the horse shoe and closer to the terminal where the interaction with MD-2 is expected, co-crystallised and present in the original pdb file (2Z64). Light blue – TLR4; dark blue MD-2 Crystal Structure; Brown – MD-2 docked structure.

In the first attempt to dock MD-2 with TLR4, all of the oligosaccharides found in the original pdb file were removed. This resulted in a best fit that showed MD-2 interacting with TLR4 on the inside of the horse shoe (Figure 5.6A brown) rather than sitting on top of the TLR4 extra cellular domain. The structures saved by the Patchdock were

modified and did not allow direct comparison with the crystal structures and RMSD calculation. However, the visual inspection revealed that the change of docking target to the TLR4 possessing all of its oligosaccharides produced a good agreement between the crystal structure and the docking result (Figure 5.6 B). Final experiment was performed keeping only the oligosaccharides inside the horse shoe showing that the docked MD-2 overlays the MD-2 in crystal structure indicating that presence of sugar molecules inside the horse shoe is fundamental for the correct positioning of MD-2 in the complex (Figure 5.6C). These validation studies confirmed that Patchdock should be suitable for further studies; however the target molecules have to be correctly defined.

The Hex software package is developed for protein and nucleic acids interaction studies. It also allows modelling of small ligand-protein interactions using rigid docking. Hex is a fast Fourier transform (FFT) docking correlations based programme that uses soft polar Fourier correlations to minimise the computational time required to explore the Cartesian space^{219, 220}. The protein's molecular surfaces are represented by an internal and an external "skin" that are each one represented by a Fourier series and comprise radial and spherical harmonic basis functions²¹⁹. Electrostatics contribution is optional but when present, it is only taken into account in the final search²²¹ and has a small weighting in the final scoring function. The resulting structures are ordered from lowest to highest energy and the structures are then clustered with a 3Å threshold for the main chain $C_{g} - C_{g}$ RMSD values²²¹.

The same crystal structure of the mouse TLR4-MD-2 complex (2z64) was used to evaluate if the software could reproduce the structure of a complex. The influence of the shape only parameters versus shape and electrostatics parameters were also assessed. Since Patchdock revealed the importance of the presence of the oligosaccharides inside TLR4's horse shoe, these were considered in the docking process with Hex.



Figure 5.7 Results obtained for the interaction study of mTLR4 extracellular domain and mMD-2, using Hex 5.0. A - Crystal structure from the mouse complex TLR4-MD-2 (2Z64); B - Docking results obtained for the mouse complex TLR4-MD-2 taking into account both shape and structure; C - Hex 5.0 docking results for the mouse complex TLR4-MD-2 with a shape only protocol. For A and B: Light blue – TLR4; dark blue MD-2 Crystal Structure; Brown – MD-2 docked structure. For C: Green – TLT4; Pink – MD-2. The saccharide molecules in TLr4's horse shoe were hidden for clarity.

The docking results of the mouse TLR4-MD-2 complex show that the Hex shape only protocol is not enough for the software to describe the system correctly (Figure 5.7 C). If a contribution from electrostatics, even with a smaller weighting, was taken into account, the docking reproduced the crystal structure with a small deviation but

maintaining the correct interaction site of MD-2 with TLR4. Therefore, the Hex shape and electrostatics protocol was considered suitable for the study.

Finally, for a better understanding of the electrostatics contribution in the interaction of the dendrimers with the LPS recognition system, GRID software was considered.

GRID²²² is a calculation based procedure that allows one to determine the energetically favourable binding sites on a molecule with a known structure. GRID results can be visualized with the Gview application. This application allows visualization of molecular interaction fields, GRID energy contributions due to atoms of the target and molecular structures with distances, torsion and dihedral angles. Glue is a GRID-docking programme capable of finding possible interaction sites between a molecule set as "target" and a small molecule called ligand. It requires the input of both the target and the ligand 3D structures. Its scoring function takes into account steric repulsion energy, electrostatics contribution, a dry parameter which accounts for hydrophobic energy and an additional hydrogen bonding charge reinforcing parameter.

The system used for Grid validation was MD-2-Lipid IVA (2z59). The results are shown in Figure 5.8.



Figure 5.8 GRID protocol validation. A - Crystal structure of the human MD-2-Lipid IVA complex (2e59); B structure obtained with Glue for the same complex.

Noting the structure of the MD-2-Lipid IVA complex, it appears the GRID results are very similar to the crystal structure in terms of Lipid A conformation and position within the MD-2 cleft. Based on these results GRID was considered suitable for the further study.

With the completion of these preliminary validation experiments, these three software packages were selected and further evaluated for the interaction studies with dendrimers. One, using a purely geometric based algorithm (Patchdock), one using a shape based algorithm but taking into account small electrostatic contributions (Hex), both of these dedicated to protein-protein interaction. Finally, a package considering electrostatics contributions (Glue), within the GRID protocol was selected.

Electrostatic contribution for the interaction of gen. 3.5 PAMAM dendrimers and the MD-2 protein

The interactions between the dendrimers and the MD-2 protein were investigated using GRID protocol and validated as described above. The study started with the docking of small glucosamine molecules and the MD-2 protein (Figure 5.9). Maltose and sucrose were also docked against MD-2 protein as negative control (Appendix 5.1).



Figure 5.9 Three representative interaction sites of glucosamine and MD-2 (A, B and C) as predicted by molecular docking. This docking was performed using GRID software. Two glucosamine molecules were represented in each structure.

From the results there seems to be no specificity for the interaction between free glucosamine and MD-2. The glucosamine molecules seem to position to the opposite sides of the opening of the cavity as is found in the crystal structure of MD-2 in complex with lipid IVa (2E59) (Figure 5.7 B). The glucosamine also is found at the opening showing a clear interaction with either side of the opening. They appear to be almost "floating" in front of the cavity (Figure 5.7 A and C). These results are consistent with previously unpublished studies from Prof. Shaunak's group that show that it is possible for glucosamine to have some inhibitory effect on the LPS triggered inflammatory response in vitro if added in extremely large quantities. These results are also consistent with the results described in Chapter II where the saccharide used to modify the dendrimer was changed. In this case, the LPS triggered inflammatory response was

again inhibited which may also be construed that there is non-specific interaction between MD-2 and sugars.

The study then proceeded to evaluate dendrimers with increasing branch sizes to represent different patterns of the dendrimer glucosamine surface. These experiments were done with selected parts of the dendrimer from the representative structures obtained after a 4.8 ns molecular dynamics simulation that had been preformed with Desmond (refer to chapter IV for details).



Figure 5.10 Interaction between MD-2 and different branched molecules representing selected patterns of the dendrimer glucosamine surface. A – docking results of MD-2 with a branch representing the last half generation of dendrimer glucosamine with only one glucosamine molecule; B - docking results of MD-2 with a branch representing the last generation of dendrimer glucosamine with two glucosamine molecules placed in both extremes of the ramification; C - docking results of MD-2 with a branch representing the last generation of dendrimer glucosamine with two glucosamine molecules one placed in the extreme of the ramification and the other on the internal face of the ramification.

The interaction between MD-2 and the smallest dendrimer branch with glucosamine conjugated is similar to the interaction between MD-2 and glucosamine alone. All solutions of the docking indicate that the molecule faces the cavity but there is no specific interaction with either side of MD-2 cavity (Figure 5.10 A). As the size of the

branches increases there is an increased flexibility in the molecule that allows an increased interaction of the saccharides (at least two) with both sides of the cavity (Figure 5.10 B and C). While these experiments are preliminary, they support the idea that the opening of MD-2 may be a potential interaction site of the dendrimer glucosamine with the LPS recognition system.



Figure 5.11 Interactions of ligands with the opening of MD-2 in A – Crystal structure of MD-2 complexed with Lipid IV A; B – docking solution of a small dendrimer branch with two glucosamine residues. The atoms/residues of the MD-2 that are in close proximity to ligand (less than 4 Å) are represented in yellow in both structures.

The comparison between the possible sites of interaction for Lipid IV A and the dendrimer revealed that both molecules are capable of developing interactions with the opening of the MD-2 cavity (Figure 5.11). In some cases the interacting amino acid residues are the same, for example Ser118. It was also revealed that both the branching units and the glucosamine molecules can develop interactions with these residues in the entrance of MD-2 pocket. Finally, the results showed the dendrimer could interact with both sides of the opening, thus blocking its entrance.

These docking studies are not definitive for various reasons. First, the portions of dendrimer used are not representative of entire structure of the dendrimer due to their

size and the low probability that two glucosamine molecules will be found on the same branch (as discussed in Chapter IV). These assumptions had to be considered since the software cannot handle the whole molecule. Secondly, a closer inspection of the resulting structures on Figures 5.10 and 5.11 reveal that the branching point of the dendrimer is pointing towards the inside of MD-2 pocket, which would not be possible due to the dendrimer large size and hydrophilic nature. Some sections of the surface of the dendrimer portions are hydrophobic, while as a matter of the fact, the surface of the whole dendrimer is hydrophilic. Given the limitations of the software to use whole dendrimer, this line of study was abandoned. Nonetheless, the results of the glucosamine molecule interaction studies with MD-2 revealed potential places for interaction and hence it was hypothesised that "hot-spots" could be established for the whole TLR4-MD-2 complex.

In order to investigate this possibility, docking of the glucosamine molecules with sequential sections of the human TLR4-MD-2 (3FXI) complex were performed with Glue, which is part of the GRID programme (Figure 5.12). The complex was set to be the target for docking in sections. Several docking experiments were conducted to increase the definition within each grid and the results were classified into four categories according to their interaction energies expressed in kcal/mol.



Figure 5.12 Solutions of the docking studies between the TLR4-MD-2 complex and glucosamine, performed with GRID protocol. A - Top view from the TLR4-MD-2 complex with all possible sites for interactions with glucosamine molecules; B - Bottom view from the TLR4-MD-2 complex with all possible sites for interactions with glucosamine molecules; C - MD-2 opening view from the TLR4-MD-2 complex with all possible sites for interactions with glucosamine molecules; C - MD-2 opening view from the TLR4-MD-2 complex with all possible sites for interactions with glucosamine molecules. Red – Energy values (kcal/mol) lower than -8; Green – between -5 and -8; Black – between -2 and -5; Pink – between 0 and -2.

The raw results for the docking between the TLR4-MD-2 complex and glucosamine were filtered to allow for the identification of "hot spots" for interaction. All solutions at the C-terminal of TLR4 were discarded since this is the region where anchoring to the cell membrane is thought to occur¹⁸⁴. Since the glucosamine molecules are small, the energy of interactions are low and it can be difficult to establish energy cut off values as criteria to identify potential interaction sites. However, the docking solutions with interaction energies between 0 and -2 kcal/mol were discarded as well as potential places for interaction with single glucosamine molecule. After discarding these solutions, it was possible to have more clear view of the potential sites for glucosamine interaction (Figure 5.13).



Figure 5.13 Solutions of the docking studies between the TLR4-MD-2 complex and glucosamine, performed with GRID protocol. A - Top view from the TLR4-MD-2 complex with the "hot spots" for the interactions with glucosamine molecules; B - Bottom view from the TLR4-MD-2 complex with the "hot spots" for the interactions with glucosamine molecules; C - MD-2 opening view from the TLR4-MD-2 complex with the "hot spots" for the interactions with glucosamine molecules; B - C - MD-2 opening view from the TLR4-MD-2 complex with the "hot spots" for the interactions with glucosamine molecules. Blue – TLR4-MD-2 complex structure, represented as ribbons; Red- interacting protein residues and glucosamine molecules; the glucosamine molecules are represented as CPK, the size of CPK model of each spot is scaled by the number of molecules and energy of interaction with the proteins (0.9 for small spots, 1.7 for medium spots and 2 for large spots).

Interestingly, the two most significant interaction "hot spots" on the receptor surface for the interaction of glucosamine molecules are located in site of interaction between MD-2 and TLR4 and on one side of the opening of MD-2 (Figure 5.13 C). Additionally, four other spots were identified that were dispersed along TLR4 horse shoe, and a small one

in the opposite side of MD-2 cavity (Figure 5.13 A and B). The results were encouraging. Despite the non-specific nature of the interactions, there are several tentative "hot spots" where the glucosamine moieties of glycosylated dendrimer could interact with MD-2 and disrupt TLR4-MD-2-LPS complex formation or receptor dimerization. Key finding is that there are two strong hot spots around the anchoring site of MD-2 to TLR4 and MD-2 itself.

Shape based interaction of gen. 3.5 PAMAM dendrimers and the MD-2 protein

For this study 20 representative conformations of each PAMAM dendrimer were selected and submitted as ligands to both Patchdock and Hex. The target used was the crystal structure of human MD-2 (2E59). For each ligand the 20 lowest energy solutions were saved (a total of 400 solutions per dendrimer). To process this data a rebol script available in the Structural Chemistry group at the School of Pharmacy was adapted to this system (Appendix 5.2) and was run with Vega ZZ interface.

The results were analysed in terms of the number of interactions between atoms in the dendrimer and the MD-2 residues, for both the generation 3.5 PAMAM dendrimer (inactive) and the glycosylated generation 3.5 dendrimer (active) (Figure 5.14).



Figure 5.14 Interaction studies between PAMAM dendrimers and MD-2 protein, performed with Patchdock. Green – generation 3.5 PAMAM dendrimer modified with eight glucosamine molecules; Blue – generation 3.5 PAMAM dendrimer without any modification.

In general, both dendrimers revealed similar profiles though the glycosylated dendrimer develops a higher number of interactions with MD-2 then the non-modified dendrimer. This indicates that the presence of the conjugated saccharides on the dendrimer surface potentiates the interaction with MD-2 accessory protein (Figure 5.14). The residues between 117 and 127 of MD-2 were of particular interest as they are located in the opening of the cavity. This again is consistent with the hypothesis that the glycosylated dendrimer may exert it biological properties by blocking the MD-2 pocket to prevent LPS binding. However, the difference between the two dendrimer results can only be seen as trend and these possible interactions required further study.

To investigate if there is a specificity of the interactions observed with Patchdock, the solvent accessible surface area of each residue of MD-2 was determined and plotted along with the number of interactions for the glycosylated dendrimer (Figure 5.15 top). Comparison of these two profiles indicated that the most solvent exposed residues are not always the ones with highest number of interactions. This was a good indication that there is some degree of selectivity in the interactions between the glycosylated dendrimer and MD-2. Such selectivity was evaluated by dividing the number of observed interactions by solvent accessible area (SASA), which indicated areas that may be important for the dendrimer biological relevance, namely residues from 98 to 117 around the MD-2 opening (Figure 5.15 bottom).



Figure 5.15 Comparative studies between the availability of the residues and the number of interaction of these with glycosylated dendrimer. Top: Green – Interaction studies between generation 3.5 PAMAM dendrimer modified with eight glucosamine molecules and MD-2 protein, performed with Patchdock.; Red – Solvent accessible surface area (SASA) determined for each residue of the MD-2 protein. Bottom: Number of interactions between the glycosylated dendrimer and the residues of MD-2 normalised to the availability of the protein residues.

In summary, the preliminary experiments with Patchdock indicate that there is some selectivity in the interaction of the glycosylated dendrimer with MD-2. Although it was not possible to distinguish unique sites of interaction for the glycosylated dendrimer compared to those of the non-modified dendrimer, it is apparent that the presence of the

glucosamine on the dendrimer surface potentiates the interactions of the dendrimer with MD-2.

The same conformations of both dendrimers were than submitted for docking with the Hex shape and electrostatics protocol. Initially, the results were analysed in terms of number of interactions between atoms on the dendrimer and the MD-2 residues. Both the generation 3.5 PAMAM dendrimer (inactive) and the glycosylated 3.5 generation dendrimer (active) were used again (Figure 5.16).



Figure 5.16 Interaction studies between PAMAM dendrimers and MD-2 protein, performed with Hex. Green – generation 3.5 PAMAM dendrimer modified with eight glucosamine molecules; Blue – generation 3.5 PAMAM dendrimer without any modification.

The results obtained with Hex, were not significantly different from those obtained with Patchdock, with both dendrimers showing the same type of interaction profile. However, a larger number of interactions were detected with HEX, between both dendrimers and the MD-2 residues, indicating that the electrostatic contribution is relevant for the interaction. Furthermore, in the region of the opening of the MD-2 cavity, the difference between the glycosylated dendrimer and the non modified dendrimer is even more evident (Figure 5.16, residues 91 to 122).

To inspect the differences between the two software packages for each dendrimer, the results of the interactions for each dendrimer with MD-2 were compared (Figure 5.17).



Figure 5.17 Interaction studies between PAMAM dendrimers and MD-2 protein, performed with Hex and Patchdock. Top – generation 3.5 PAMAM dendrimer without any modification; Bottom - generation 3.5 PAMAM dendrimer modified with eight glucosamine molecules.

This comparison again supports the importance of the electrostatic contribution, and when this contribution is taken into account the number of interactions increases for both dendrimers. A closer look at the results for the glycosylated dendrimer (Figure 5.17

bottom) revealed that the electrostatic contribution largely increased the number of interactions with residues 91 to 122. These are all located in the opening of the cavity of MD-2.

Although these were encouraging results to support the hypothesis of the pathway being disrupted by blocking the MD-2 pocket, both dendrimers did display a high number of interactions. However, it is known that only the glycosylated dendrimers are capable of disrupting the inflammatory response (data not published from Prof. Shaunak's group). Thus it was important to distinguish which interactions might be in fact relevant for the observed biological activity. For this purpose, the profile of the PAMAM dendrimer on its own was subtracted to the one of the glycosylated PAMAM dendrimer, as obtained from both software packages (Figure 5.18).



Figure 5.18 Difference between the number of interaction with MD-2 of the gen.3.5 PAMAM dendrimer and the glycosylated gen. 3.5 PAMAM dendrimer. Pink – docking study performed with Hex. Blue - docking study performed with Patchdock.

Patchdock results show that the glycosylated dendrimer has a superior number of interactions compare to non-glycosylated dendrimer for all MD-2 residues. The profile obtained with Hex results showed some remarkable results. This profile revealed that

there are more interactions between the non-glycosylated dendrimer with the residues located on the sides and back of MD-2 protein. In contrast, the glycosylated dendrimers show a much higher number of interactions with the opening of MD-2 pocket. This suggested that these are relevant interactions that may be involved with discriminating the active and non-active forms of the dendrimer.

These results were the first to indicate a possible mechanism of interaction for the biological activity of the glycosylated dendrimers. However, this analysis only considered the number of possible interactions without considering their relative binding energies. The interactions found with Hex were then normalised with the energy value calculated for each solution.



Figure 5.19 Interaction studies between PAMAM dendrimers and MD-2 protein, performed with Hex, normalised with the energy value of each interaction. Green – generation 3.5 PAMAM dendrimer modified with eight glucosamine molecules; Blue – generation 3.5 PAMAM dendrimer without any modification.

When energy was taken into account the differences between the dendrimer without modifications and the glycosylated dendrimer became more evident (Figure 5.19). The active glycosylated dendrimer revealed both a greater number and stronger interactions with the MD-2 residues 57-67 and, more importantly, between residue 91 and residue

122 located in the opening of MD-2 pocket where LPS binds. To allow the identification of the interactions relevant for the biological activity, specifically, in the region of the entrance of MD-2 pocket, the profile of the PAMAM dendrimer on its own was subtracted from the profile of the glycosylated PAMAM dendrimer.



Figure 5.20 Difference between the number of interaction with MD-2 of the gen.3.5 PAMAM dendrimer and the glycosylated gen. 3.5 PAMAM dendrimer, from the docking study performed with Hex and normalised with the energy value of each interaction. The graph shows only the residues in the opening of MD-2 cavity.

The analysis of the difference between the active and the inactive dendrimers revealed the importance of specific residues for the biological role of the glycosylated dendrimer (Figure 5.20). The most relevant residue is Tyr102, followed by Lys91, Arg96, Arg106, Asn114 and Ser118. Other residues, with lower interaction values but that might also contribute to binding with the glycosylated dendrimer, are Gly97, Lys109, Thr112 and Thr116. These residues and/or residues in their close proximity had already been identified by different authors as having a role in LPS recognition^{184, 212, 213} (Figure 5.21).



Figure 5.21 Human MD-2 crystal structure (2E56) displayed in light blue, with the residues in the opening of its cavity displayed in dark blue. A – In green the relevant residues for LPS binding and complex dimerisation, identified by other authors; B – In red residues relevant for the glycolysed dendrimer interaction with MD-2, in pink other residues that might play a role in these interactions.

In 2009, Park et al reported a study that aimed to elucidate the structural basis of LPS recognition by the TLR4-MD-2 complex¹⁸⁴. In this study some residues on MD-2 protein were identified as crucial for the interaction with LPS and for TLR4-MD-2 complex dimerisation. The authors identified MD-2 residues Phe126, Arg90 and Leu87 as involved in receptor dimerisation and a hydrogen bond between the phosphate in the saccharide residue of LPS and MD-2 Ser118¹⁸⁴ (Figure 5.21 A) where the saccharide portion of LPS or TLR4 may interact with MD-2. A year later Meng et al, investigated ionic interactions between Lipid A and TLR4 that are mediated by MD-2. For the purpose of this study the relevant human MD-2 residues identified were Cys95 and Cys105 which were shown to interact with the phosphates from Lipid A and also the residues Lys122, Lys125 and Phe126 which are crucial for complex dimerisation²¹³ (Figure 5.21 A). These residues are positively charged in human MD-2 but not in mouse MD-2. The authors propose the charge inversion has consequences on the binding and

accommodation of the acyl chains of Lipid IVA, resulting in this molecule no longer acting as agonist²¹³. More recently, the same authors showed that the MD-2 residues Tyr42, Arg69, Asp122 and Leu125 provide species-specificity response to Lipid IVa²¹².

This PhD is focused only on the interactions of the glycosylated dendrimer with the human LPS recognition system, more specifically with the hMD-2 protein, and how these disrupt the LPS triggered inflammatory response. The residues identified with these docking studies revealed that the glycosylated dendrimer has higher affinity for residues close but not exactly the same as the previously identified for LPS binding and dimerisation with the exception of Ser118 (Figure 5.21 B). Nonetheless, these results further support the hypothesis that the glycosylated dendrimer disrupts the LPS triggered inflammatory response by binding to the MD-2 opening and thus competing with LPS binding.

To assess the dynamic behaviour of these interactions and to evaluate if the atom type parameters of the protein-protein docking software were affecting the results, a 4.8 ns molecular dynamics simulation of the resulting complex from docking between the glycosylated dendrimer and MD-2 obtained with Hex was performed. It is acknowledged that much longer simulations are currently being performed with dendrimers and large systems, however with the computational power available performing longer simulations would not be viable.



Figure 5.22 Six structures representing the snapshots of molecular dynamics simulation of the glycosylated dendrimer with MD-2 obtained with Hex. Top left to bottom right: 0 ns; 1.6 ns; 2.4 ns and 4 ns. The arrow highlights MD-2 pocket. MD-2 is displayed as ribbons, the glycosylated dendrimer as tube and the glucosamine molecules on the surface of the dendrimer as CPK.

In the Hex docking solution structure, at the initial starting point of 0 ns, the glycosylated dendrimer is positioned in front of the cavity with three glucosamine molecules in close proximity of the MD-2 protein (Figure 5.22 top left). As the simulation progresses the dendrimer moves slightly away from MD-2 and rearrange its

branches so there are more glucosamine molecules facing the protein. From the middle (2.4 ns) to end of the simulation, the dendrimer rearranges in such a way that the glucosamine molecules seem to get closer and closer as time progresses and make close contact with MD-2, by the 4 ns five glucosamine molecules seem to embrace MD-2 (Figure 5.22 and 5.23). An additional simulation was performed where the surface of the dendrimer facing the MD-2 had no glucosamine molecules conjugated. The negative charge on the dendrimer surface did not show favourable interaction, so the dendrimer moved away from MD-2 during first 400 ps of the simulation. Furthermore the glycosylated dendrimer underwent the conformational change so that the branches bearing the glucosamine molecules faced MD-2 to form favourable interactions and moved back getting closer to MD-2 opening (Appendix 5.2)


Figure 5.23 Surface contact area between MD-2 protein and the glycosylated dendrimer at: A - 0 ns; B - 1.6 ns and C - 2.4 ns. Left – frontal view; Right – side view. The MD-2 surface is represented in grey, the contact surface is represented in green and the glycosylated dendrimer surface is represented in pink with 80% transparency.

The contact surface area analysis revealed the changes that occurred during the simulation. Initially there is a larger contact between the dendrimer and the protein but from 2.4 ns the opposite tendency is observed. More importantly, despite this conformational rearrangement and the variation in the contact area, the pocket of MD-2 is covered by the dendrimer at all times. This is visible in Figure 5.23 by the pink colour of the pocket which indicates that the dendrimer is in front of the protein. Furthermore, the presence of the dendrimer seems to induce conformational changes on MD-2 protein, namely in the entrance of the cavity.



Figure 5.24 Superposition of MD-2 protein at different time points of the molecular simulation with the glycosylated dendrimer. The MD-2 is represented as ribbons for clarity and the brown arrow represents the opening of the protein's pocket.

The superimposition of the MD-2 structure (Figure 5.24) at three different time points of the simulation showed that there are significant changes from one structure to the other. These differences appear to have an impact on the shape of the entrance making it

narrower with time. A large shift also seems to be in the region where the interaction with the dendrimer happens. This suggests that the presence of the dendrimer is directly related to these conformational changes. This observation suggests that in the event of competition between the dendrimer and LPS, the dendrimer would induce conformational changes in MD-2 that most likely would make LPS access to the pocket more difficult. However, this could only be assessed with docking experiments between LPS and MD-2 in the new conformation and molecular dynamics simulations where all three molecules were present.

This approach in molecular docking has shown a possible mechanism for the interaction between the glycosylated dendrimer and the MD-2 protein. Initially, the dendrimer interacts with MD-2 in the entrance of its pocket. This can prevent access of LPS access to the hydrophobic cavity of MD-2. This model is also valid for the free form of MD-2 circulating in the blood stream. However, as mentioned, there is no direct evidence if LPS binds to MD-2 in the blood stream or to the MD-2-TLR4 complex. The lack of a crystal structure of the MD-2-TLR4 complex without bound LPS and the suggestion of the complex as not being stable without LPS by Vistidin and co-workers, suggested that this is a mechanism which best fits with what is currently known.

In the event of MD-2 first being complexed with TLR4, the model based on these docking results may no longer be accurate, since the residues with which the dendrimer seems to interact with higher specificity are the same ones previously shown to be involved in the interactions between MD-2 and TLR4. Hence these docking studies support the idea that LPS may bind to the MD-2 prior to TLR4 complexation.

Due to the size of the system it would be difficult to repeat the same experiments and data analysis with the whole system. Hex software however, seemed able to cope with the system size and twenty representative structures of the glycolysed dendrimer were docked against the TLR4-MD-2 complex. The results showed different hot spots with different possible places for interaction. Nevertheless, some solutions are consistent both with the pseudo-pharmacophore and the findings of the docking of the whole glycosylated dendrimer with MD-2 (Figure 5.25).



Figure 5.25 Docking solution between the TLR4-MD-2 complex (ribbons) and the glycosylated PAMAM dendrimer ²²³, obtained with Hex software. A – Side view of the resulting structure; B – Frontal view of the resulting structure; C – Detail of the frontal view of the resulting structure. The MD-2 protein is represented in blue and the previously identified residues are coloured in red and pink as in figure 5.14.

This solution revealed the glycosylated dendrimer seems to approach the complex form the side of TLR4 opposite to where MD-2 is bound and that three of its glucosamine molecules are close to the residues identified in the studies with MD-2 as the potential sites for interaction with the dendrimer. This solution not only supports the previous study as it allows for speculation of an alternative mechanism of action for the glycosylated dendrimers, where the dendrimer interacts with both components of the complex preventing dimerisation and/or causing conformational changes in MD-2 that prevent LPS binding to the complex. For a better understanding of this possible alternative mechanism a simulation of this solution should be performed.

Conclusion

A range of software packages were evaluated where it was found that three packages could be used to study the interactions of the glycosylated dendrimer with the MD-2 protein and the TLR4-MD-2 complex. The docking studies performed with the three selected software packages indicated a possible mechanism for the interaction of the dendrimer glucosamine with the MD-2 accessory protein. The docking of only glucosamine molecules with the TRL4-MD-2 complex revealed a series of "hot-spots", allowing the detection of probable sites of interaction. The docking software based on shape complementarity allowed the study of the whole dendrimer with MD-2, leading to a potential mechanism which was consistent with the hypothesis that the glycosylated dendrimer interacts with charged residues in the opening of MD-2 pocket to block the entrance and to prevent LPS binding. The dynamic behaviour of this interaction has shown that the dendrimer undergoes conformational changes so more glucosamine molecules become closer to MD-2, maintaining the MD-2 cavity blocked at all times, furthermore, the presence of the dendrimer induces conformational changes on MD-2 causing the entrance of the pocket to become narrower. A preliminary study with the glycosylated dendrimer and the TLR4-MD-2 complex indicated that the dendrimer could interact with both components of the complex preventing dimerisation and/or causing conformational changes in MD-2 that would prevent LPS binding to the complex.

CHAPTER VI – RATIONAL DESIGN OF A GLYCOSYLATED, HYBRID TRIAZINE-PAMAM DENDRIMER CAPABLE OF BLOCKING TLR4 INDUCED INFLAMMATION.

Introduction

Glycolysed generation 3.5 PAMAM dendrimers have been shown to inhibit the LPS triggered-TLR4 mediated inflammatory response¹. This response is often referred to as a cascade which culminates with the release of chemokines and cytokines leading to sepsis and death¹. These glycosylated dendrimers exist as a mixture of different chemical entities and therefore are not amenable for full chemical characterisation^{65, 87}. The divergent methods used for their synthesis along with aspects of the reactions used during the synthesis (e.g. Michael additions with amine nucleophiles) contribute to the heterogeneity of glycosyl conjugation reaction, many researchers have published data indicating that the PAMAM dendrimers often have some heterogeneity associated with their structure^{65, 87}. This is often thought to be due to retro-Michael reactions. While the glycosylated PAMAM dendrimers display biological activity that has potential therapeutic benefit, there may be some limitations in using the current PAMAM dendrimer as a basis for these saccharide conjugates.

The previous chapters have shown that the biological activity of the dendrimers is related to their surface properties. As such, it was thought that a different dendrimer could be designed to obtain the same immuno-modulatory properties. There are many different dendrimers with potentially more amenable chemical properties such as greater structural homogeneity and which are much less expensive to make. A class of water-soluble dendrimer derived from triazines (Scheme 6.1, in the Results and discussion section) have been shown to be easily synthesised as a single chemical entity²²⁴ at the kilogram scale²²⁵. These triazine dendrimers provide a potential template for surface modifications towards the desired biological activity.

The triazine ring comprises both hydrogen acceptor and donor groups. Triazines are very attractive as building blocks for dendrimeric structures²²⁶. Many different synthetic routes for triazine dendrimers have been reported and they are extensively reviewed²²⁶⁻²²⁹. However for context, some of the synthetic details for these dendrimers will be briefly mentioned.

There are two main methods used for the synthesis of triazine dendrimers, which are cycloaddition and nucleophilic aromatic substitution. Both approaches can then be used in divergent or convergent synthetic strategies. Cycloaddition is used in an iterative process that is based on the addition of modified guanidines to the nitrile groups of interest (Figure 6.1). These reactions are conducted in the presence of a base at high temperatures²³⁰⁻²³⁴.



Figure 6.1 Diaminotriazine derivative formation, using cycloaddition methods²²⁶.

The aromatic substitution strategy is more commonly used and is usually achieved by a chemoselective nucleophilic attack on cyanuric chloride ($C_3N_3Cl_3$) or its alkoxy-substituted triazine derivatives²³⁵⁻²³⁹ (Figure 6.2).



Figure 6.2 Example of a convergent synthesis of a triazine dendrimer using nucleophilic aromatic substitution method, with dibutylamine as precursor²²⁶.

The potential applications of triazine dendrimers have been widely investigated in recent years, they range from medical to material science applications amongst others. In 1997 a patent was filed for the use of triazine containing dendrimeric structures (CL309623 and derivatives) as antiviral agents, specifically, against Human Repiratory Syncytial Virus (RSV)²⁴⁰. In 1998, Ding et al reported a new triazine dendrimer as an inhibitor for the same virus with even higher potency. This dendrimer had been developed as a result of a SAR study from Wyeth-Ayerst²⁴¹. A group of inhibitors resulting from a screening study was later reviewed by Gazuman et al in 2000²⁴². Finally, in 2001 compounds were optimised which resulted in the synthesis of another molecule known as RFI-641²⁴³. These molecules are thought to prevent the fusion of the virus with the cell²⁴¹. RFI641 was reported to have reached Phase II clinical trials, and CL309623 to progress to lead discovery or lead optimization stage at Wyeth. However both compounds were not found on active R&D projects on the company's website since 2003, suggesting they were discontinued²⁴⁴.

Triazine-based compounds with antibacterial activity were found by screening of single-bead constructs made of dendrimers bound to a resin which were then modified with a library of triaze derivatives²⁴⁵.

The Simanek group has reported the use of various triazine dendrimers as vehicles for drug delivery. In 2004, this group published toxicity studies with melamine dendrimers showing their toxicity levels were comparable with cationic PAMAMs and establishing that the surface groups were determinant factor for toxicity and consequentially for their use in drug delivery²⁴⁶. In 2009, they reported a general strategy for the use of triazine dendrimers as delivery systems which showed promising *in vitro* results²⁴⁷. Two other studies were described with different triazine constructs conjugated with paclitaxel^{248, 249}. Initially, they presented a successful synthesis strategy and characterization of a melamine dendrimers with paclitaxel suitable for drug delivery use. Consequently, a complete study on the design, synthesis, characterization and biological evaluation (*in vitro*) of three triazine dendrimers paclitaxel conjugates with tolerable toxicity levels and tumour selectivity was described. Unfortunately the blood circulating half-lives were observed to be too short²⁴⁸. More recently, in 2010, the group published *in vitro* and *in vivo* studies on PEGylated triazine dendrimers with paclitaxel

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showing that they remained biologically active, with an *in vitro* cytotoxicity 2 to 3 orders of magnitude lower than Taxol[®] and with *in vivo* toxicity comparable to Abraxane in prostate tumor model (PC-3-luc)²⁵⁰. However some issues remained that mainly related to dendrimer aggregation²⁵⁰. The same group has also suggested the use of triazine dendrimers for gene transfection^{251, 252}. Other interesting potential applications of triazine dendrimers comprise columnar liquid crystals²⁵³, phosphorescent Organic Light emitting diodes²⁵⁴, tectons²⁵⁵ and surfactants²⁵⁶.

Our interest in these dendrimers became evident after Simanek group reported a divergent synthetic strategy that allowed the synthesis of a generation two triazine dendrimer as a single entity (MALDI-TOF analysis) at the kilogram scale²²⁵. Furthermore, the same group reported later the synthesis and characterisation of generation two and three triazine dendrimers with carboxylic acid end groups²²⁴. In a comparative study, Lalwani et al. characterised by capillary electrophoresis generations two and three triazine dendrimers and a generation 1.5 PAMAM dendrimer and a generation 2 succinate PAMAM dendrimer, showing that the triazines exist as a single entity and while the PAMAM dendrimers were a heterogeneous mixture²²⁴. From the perspective of structural purity and ease of synthesis, these findings made triazine dendrimers good candidates for glycosylation and subsequent biological evaluation. However, preliminary unpublished studies showed the glycosylation of generations two and three triazine dendrimers did not display any activity in the inhibition of the LPS mediate cytokine release. Since molecular modelling techniques have contributed to a better understanding of the generation 3.5 PAMAM dendrimer properties and interaction with putative biological targets, the triazines provided an opportunity to examine modelling methodologies used in this work in more detail. It was aimed to use such information and techniques to rationally design better and more potent dendrimers with preserved biological activity.

Our modelling approach was driven by the observed biology to compare a known active dendrimer glucosamine (generation 3.5 PAMAM) and a known inactive dendrimer glucosamine (generation 2 and 3 triazine dendrimers). The process involved generating 3D structures of these molecules, followed by performing implicit solvent simulations. The molecular properties were then determined to allow comparison.

Using this information a series of hybrid dendrimers that would replicate the PAMAM surface were designed and submitted to the same protocols. The hybrid triazine-PAMAM glycosylated dendrimers were then synthesised and biologically evaluated *in vitro*.

Experimental Section

3D structure generations of triazine and hybrid dendrimers

The structure of the glycosylated generation 3.5 PAMAM dendrimers was generated as described on Chapter III. In an effort to more quickly produce structural building blocks and to ensure that modelling efforts could effectively inform synthetic efforts, 2D structures of the dendrimers were built in ChemBioDraw Ultra 11.0 (ChemBioOffice 2008). These were then converted to 3D ChemBio3D 11.0 (ChemBioOffice 2008) and saved as mol files. The mol files were submitted to 3D optimization in Avogadro 1.0²⁵⁷ using MMFF force field²⁵⁸ and saved in a pdb format.

Minimization of the 3D structure of PAMAM, triazine and hybrid dendrimers

All resulting structures of the dendrimers under study were imported into Maestro version 9 (www.schrodinger.com) and minimized with Macromodel version 9.7.211¹⁵¹ for 2500 iterations using the OPLS2005 force field¹⁵⁰. The effects of water as solvent were taken into account by Generalized Born/ Surface Area (GB/SA) implicit solvent model¹³⁴. The protonation state of the nitrogen atoms in branching points and of the carboxylic groups on the surface were confirmed by pKa calculations using Sparc online version 4.5²⁵⁹.

Minimization and molecular dynamics simulations glucosamine modified PAMAM, triazine and hybrid dendrimers

Molecular dynamics simulations using OPLS2005 force field (Jorgensen, 2205) and Generalized Born/ Surface Area (GB/SA) model¹³⁴ as implicit solvent method were performed at 300 K using Macromodel. The extended cut-off distances were set at their default values for implicit solvation, specifically van der Waals (8.0 Å), electrostatic (20.0 Å) and H-bond interactions (4.0 Å). A 100 ps equilibration simulation was preceded by 2500 steps of minimization. A 2 ns production run was performed and sample structure was recorded for every 10 ps of the simulation for trajectory analysis.

Trajectory analysis and properties determination

The molecular surface was calculated for each structure in trajectories created by Maestro 9 using a 1.4 Å probe. The values were exported in "cvs" format and the image of the surface area of each dendrimer under study was exported as "jpeg" file. Flexibility was assessed with RMSD trajectory analysis where the first 20 structures of each trajectory were discarded and the RMSD determined in relation to the first of the remaining structures using Maestro toolkit and exported in "cvs" format. The hydrophobic and hydrophilic surfaces were calculated for a single structure of each trajectory using Maestro 9 with iso-values of 0.5 and -6, respectively. The images were exported as "jpeg" files and the area values were noted. The trajectories of each molecule were saved in "mol2" format and imported into VegaZZ 2.2.0.54¹⁷⁶ where the trajectory analysis tool was used for polar surface calculations along the trajectory with a probe radius of 1.4 Å. Microsoft Excel was used to plot all properties determined.

The synthesis and biological evaluation of these compounds were performed by collaborators in different laboratories. The synthesis of the triazine and hybrid dendrimers was performed by Prof. Simanek group in Texas Christian University and the surface modification with glucosamine as well as the biological evaluation, of all dendrimers, was performed at Imperial College by Prof. Shaunak group. For this reason the methods used will not be described but can be found in Appendix 6.1. For the proof of concept purposes the biological activity results will be presented in the results and discussion section.

Results and Discussion

"The author herein acknowledges the contribution from Dr. Ian Teo and Prof. Simanek's group for the synthesis and biological evaluation of the triazine-PAMAM hybrid dendrimers."

The heterogeneity inherent to PAMAM dendrimers^{65, 87, 224} detracts from their usefulness as drugs. Triazine dendrimers which can be synthesised as pure chemical entities in a much more cost-effective manner^{224, 225} may have more potential for development into pharmacologically active molecules. However, glycosylated triazine dendrimers up to generation three have shown to be inactive against TLR4 mediated inflammatory response (data not shown). To examine computationally if it might be possible to determine why the two dendrimer systems displayed such different biological properties, it was first necessary to compare the molecular properties of PAMAM and triazine dendrimers (Figure 6.3, structures 1 to 3, respectively). After their comparison, new molecules with a triazine, were designed and evaluated according to relevant criteria (Figure 6.3, structures 4 to 7).



Figure 6.3 2D structures of the glycosylate ³ drimers under study. 1 – generation 3.5 PAMAM; 2 – generation 2 triazine; 3 - generation 3 triazine; 4 – generation 2 triazine 0.5 PAMAM; 5 - generation 2 triazine 1.5 PAMAM; 6 – generation 3 triazine 0.5 PAMAM; 7 - generation 3 triazine 1.5 PAMAM.

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Figure 6.3 cont. 2D structures of the glycosylated dendrimers under study. 1 – generation 3.5 PAMAM; 2 – generation 2 triazine; 3- generation 3 triazine; 4 – generation 2 triazine 0.5 PAMAM; 5 - generation 2 triazine 1.5 PAMAM; 6 – generation 3 triazine 0.5 PAMAM; 7 - generation 3 triazine 1.5 PAMAM.



Figure 6.3 cont. 2D structures of the glycosylated dendrimers under study. 1 – generation 3.5 PAMAM; 2 – generation 2 triazine; 3- generation 3 triazine; 4 – generation 2 triazine 0.5 PAMAM; 5 - generation 2 triazine 1.5 PAMAM; 6 – generation 3 triazine 0.5 PAMAM; 7 - generation 3 triazine 1.5 PAMAM.

Evaluation of the loading and distribution of the glucosamine molecules on the surface of the triazine and hybrid dendrimer models followed the same principles applied to the active PAMAM dendrimers. As described in the Chapter IV, the most energetically and sterically favourable configurations were those with two glucosamine molecules per dendrimer branch. This provided a total of eight glucosamines on gen 3.5 PAMAM dendrimers. To achieve an analogous loading it was decided that since triazine dendrimers present three branches, that six glucosamine molecules could be used for the triazine and hybrid dendrimers.

The glycosylated PAMAM, triazine and hybrid dendrimers were simulated with implicit solvation with a pre established protocol²⁶⁰. This method was chosen for being time efficient and because comparison of simulations using implicit and explicit solvation for PAMAM had shown not to be considerably different, details of this comparison can be found Appendix 6.2.

The flexibility of glycosylated PAMAM dendrimers is a key feature of these molecules for their physicochemical and probably for their biological properties (as seen in Chapters IV and V, respectively) as immuno-modulators in the TLR4 mediated inflammatory response. RMSD values for generations two and three of the glycosylated modified triazine dendrimers were determined to assess how their values correlated to the PAMAM ones (Figure 6.4).



Figure 6.4 RMSD values determined after a 2ns molecular dynamics simulation, using the first structure of the trajectory as reference for the structure of each dendrimers after 200 ps. Blue – generation 2 triazine dendrimer modified with 6 glucosamine molecules; Bordeaux - generation 3 triazine dendrimer modified with 1 glucosamine molecule; Green – generation 3.5 PAMAM dendrimer modified with 6 glucosamine molecules.

There is a clear difference between the profiles of triazines and the PAMAM dendrimers. The PAMAM dendrimers have significantly higher values of RMSD, meaning they sample a greater conformational space. However all the dendrimers studied present high RMSD values and were for that reason considered to be flexible. There is no significant difference between the two generations of triazine dendrimers.

The generation three triazine dendrimer with a single glucosamine molecule on its surface displayed a similar RMSD profile to the generation two triazine dendrimer with six glucosamine molecules. While the differences in flexibility may appear to be important due to the nature of these molecules, at this stage, flexibility could not be used solely to justify the difference in the biological activity between the compounds.

The surface area of the triazine dendrimers was also studied and lower values were observed when compared to the active PAMAM dendrimer (Figure 6.5). This means the surface available for interaction with the biological target is smaller for triazine dendrimers.



Figure 6.5 Surface area values observed during 2ns molecular dynamics simulations of , generation 2 triazine dendrimer modified with 6 glucosamine molecules (blue); generation 3 triazine dendrimer modified with 1 glucosamine molecule (dark red); and generation 3.5 PAMAM dendrimer modified with 8 glucosamine molecules (green).

Elucidation of the molecular surface of these dendrimers revealed one other interesting observation. The triazine dendrimers appear to have much more compact branches. They appeared to be almost flat from one side (Figure 6.6). In contrast the PAMAM dendrimer appears to have a more stretched structure, with extended branches and globular shape.



Figure 6.6 Graphical representation of the molecular surface of a generation 3 triazine dendrimer modified with one glucosamine molecule (blue) and a generation 3.5 PAMAM dendrimer modified with 8 glucosamine molecules (grey). A – Profile of the triazine dendrimer; B – frontal view of the triazine dendrimer; C – PAMAM dendrimer. The size of molecules is not to scale.

The polar surface area values were also determined (Figure 6.7). As expected, they followed the same trend with the surface area. However, these differences in molecular properties are still not enough to rationalise the biological properties that were observed for the two types of dendrimer.



Figure 6.7 Polar surface area values observed during 2ns molecular dynamics simulations,. Blue – generation 2 triazine dendrimer modified with 6 glucosamine molecules; Bordeaux - generation 3 triazine dendrimer modified with 1 glucosamine molecule; Green – generation 3.5 PAMAM dendrimer modified with 8 glucosamine molecules.

Our proposed mechanism in Chapter V suggests that the dendrimers interact with MD-2 protein, which contains a hydrophobic pocket where LPS binds. The proposed mechanism hypothesises that the glycosylated PAMAM dendrimers interact with the MD-2 protein to cause conformational changes and block the cavity to prevent LPS binding. These interactions are thought to be mainly hydrophilic interactions, so hydrophilic and hydrophobic surfaces of the PAMAM and triazine dendrimers were compared.



Figure 6.8 Graphical representation of the hydrophilic (blue) and hydrophobic (brown) surfaces of: A - generation 3 triazine dendrimer modified with one glucosamine molecule, B - generation 2 triazine dendrimer modified with six glucosamine molecules; C - generation 3.5 PAMAM dendrimer modified with 8 glucosamine molecules. The hydrophobic and hydrophilic surfaces were calculated with isovalues of 0.5 and -6, respectively.

There is a clear difference between the triazine and the PAMAM dendrimers. The triazines present large exposed hydrophobic surfaces while the PAMAM is a more hydrophilic and flexible molecule (Figure 6.8). This may be a key feature that is the most likely determinant affecting the mode of interaction with MD-2 and which leads to the difference in the biological effects observed for mediating the TLR4 inflammatory response.

Based on the combination of the hydrophilic surface distribution, flexibility and size of these molecules it was postulated that a hybrid structure with a triazine core but with sufficient PAMAM surface generations that would reproduce its surface properties, namely, a hydrophilic surface would potentially inhibit the LPS mediated inflammatory response. The key was to ensure there was sufficient hydrophilic area distribution. Four different hybrid structures were designed and analysed. These were built with generations two and three triazine cores and with growing generations of PAMAM from each core. Two PAMAM generations were evaluated: 0.5 and 1.5. These hybrids were glycosylated and submitted to molecular dynamics simulations with the exact same parameters as before and their surface properties determined (Figure 6.1). The RMSD analysis of the hybrid dendrimers displayed some worth noting observations (Figure 6.9).



Figure 6.9 RMSD values determined after a 2ns molecular dynamics simulation, using as reference the structure of each dendrimers after 200 ps. Top – Hybrid dendrimers with generation 2 triazine and variable PAMAM generations. Dark blue – generation 2 triazine dendrimer modified with 6 glucosamine molecules; Light blue - generation 2 triazine and 0.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Blue - generation 2 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM dendrimer modified with 6 glucosamine molecules. Bottom - Hybrid dendrimers with generation 3 triazine and variable PAMAM generations. Brown – generation 3 triazine dendrimer modified with 6 glucosamine molecules; Red - generation 3 triazine and 0.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Red - generation 3 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Red - generation 3 triazine and 0.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Red - generation 3 triazine and 0.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Brazine molecules; Orange - generation 3 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM dendrimer modified with 6 glucosamine molecules.

The hybrids with 1.5 generation PAMAM surface seem to have higher values of RMSD than the pure triazine dendrimer or the hybrid with a 0.5 generation PAMAM surface. , This translates into a greater conformational space that can be searched by the glucosamine surface groups (Figure 6.9, series blue and orange). The 0.5 PAMAM

hybrids appear to have a more restricted conformational flexibility, particularly the one with a generation two triazine core that seems to oscillate between two stable conformations (Figure 6.9 top, light blue series).

As a brief summary, the more PAMAM generations are introduced onto the triazine core the closer the RMSD values are to the active PAMAM glucosamine dendrimers. There are no significant differences in flexibility for the two triazine cores that are derived from either two or three generations. Finally, it is noted these differences in RMSD profiles may not be enough to distinguish between active and inactive dendrimers. The surface area analysis showed more dramatic differences between the hybrids profiles along the trajectory (Figure 6.10).



Figure 6.10 Surface area values determined after a 2ns molecular dynamics simulation, using as reference the structure of each dendrimers after 200 ps. Top – Hybrid dendrimers with generation 2 triazine and variable PAMAM generations. Dark blue – generation 2 triazine dendrimer modified with 6 glucosamine molecules; Light blue - generation 2 triazine and 0.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Blue - generation 2 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM dendrimer modified with 6 glucosamine molecules. Bottom - Hybrid dendrimers with generation 3 triazine and variable PAMAM generations. Bordeaux – generation 3 triazine dendrimer modified with 6 glucosamine molecules; Red - generation 3 triazine and 0.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Green – generation 3 triazine and 0.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Red - generation 3 triazine and 0.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Orange - generation 3 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Green – generation 3 triazine molecules; Green – generation 3 triazine molecules; Orange - generation 3 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM dendrimer modified with 6 glucosamine molecules;

The hybrids with a 0.5 generation PAMAM surface have similar properties to the glycosylated triazine dendrimers. The values converge towards the end of the trajectory.

However, the values of the surface area of the hybrids with 1.5 generation PAMAM are closer to the active PAMAM dendrimer. The hybrid with the generation three triazine with 1.5 PAMAM generations has higher values than the active PAMAM itself. These observations indicated an increase of the area available to interact with the biological target if 1.5 PAMAM generations were added to the triazine cores. Nonetheless, it was important to verify that the polarity of these surfaces followed the same trend (Figure 6.11).



Figure 6.11 Polar surface area values determined after a 2ns molecular dynamics simulation. Top – Hybrid dendrimers with generation 2 triazine and variable PAMAM generations. Dark blue – generation 2 triazine dendrimer modified with 6 glucosamine molecules; Light blue - generation 2 triazine and 0.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Blue - generation 2 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Green – generation 3.5 PAMAM dendrimer modified with 6 glucosamine molecules. Bottom - Hybrid dendrimers with generation 3 triazine and variable PAMAM generations. Bordeaux – generation 3 triazine dendrimer modified with 6 glucosamine molecules; Red - generation 3 triazine and 0.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Orange - generation 3 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Orange - generation 3 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Orange - generation 3 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Orange - generation 3 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Orange - generation 3 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Orange - generation 3 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Orange - generation 3 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Orange - generation 3 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Orange - generation 3 triazine and 1.5 PAMAM hybrid dendrimer modified with 6 glucosamine molecules; Orange - generation 3.5 PAMAM dendrimer modified with 6 glucosamine molecules.

Excluding the generation three triazine 0.5 PAMAM which shows even lower polar surface area in relation to the pure triazine dendrimer, the trend of increase in the profiles was maintained One possible explanation for this is since these molecules have a very hydrophobic core the branches might be folding in toward the core leaving these

molecules with a more compact structure that leads to the loss of the globular shape associated with PAMAM dendrimers (Figure 6.12 A). The same cannot be said for the hybrid with 1.5 PAMAM generations, it presents a more relaxed structure with stretched branches that resemble the PAMAM overall shape (Figure 6.12 B and C).



Figure 6.12 Molecular surface of : A - generation 3 triazine 0.5 PAMAM hybrid dendrimer modified with six glucosamines (red); B - generation 3 triazine 1.5 PAMAM hybrid dendrimer modified with six glucosamines (orange); C - and a generation 3.5 PAMAM dendrimer modified with 8 glucosamine molecules (grey). The size of molecules is not to scale.

The hydrophilic and hydrophobic surfaces have been identified as one of the determinant factors for the biological relevance of these molecules; as such these were determined for the four hybrids under study (Figure 6.13).



Figure 6.13 Hydrophilic (blue) and hydrophobic (brown) surfaces of: A - generation 2 triazine dendrimer modified with six glucosamine molecules, B - generation 2 triazine 0.5 PAMAM hybrid dendrimer modified with six glucosamine molecules; C - generation 2 triazine 1.5 PAMAM hybrid dendrimer modified with six glucosamine molecules; D - generation 3 triazine dendrimer modified with one glucosamine molecule; E - generation 3 triazine 0.5 PAMAM hybrid dendrimer modified with six glucosamine molecule; F - generation 3 triazine 0.5 PAMAM hybrid dendrimer modified with six glucosamine molecules; F - generation 3 triazine 1.5 PAMAM hybrid dendrimer modified with six glucosamine molecules; F - generation 3 triazine 1.5 PAMAM hybrid dendrimer modified with six glucosamine molecules; G - generation 3.5 PAMAM dendrimer modified with 8 glucosamine molecules. The hydrophobic and hydrophilic surfaces were calculated with isovalues of 0.5 and -6, respectively.

Noting the graphical representations for the distribution of the hydrophilic and hydrophobic surfaces, it is possible to see that the glycosylated 0.5 PAMAM hybrids follow the same trend as the pure glycosylated triazine dendrimers. Both this hybrid and the pure triazine dendrimers have large hydrophobic areas that are exposed on the surface (Figure 6.13 A, B, D and E). In contrast, the glycosylated 1.5 PAMAM hybrids seem to have the hydrophobic area more fully contained within the PAMAM portion resulting in a significantly larger hydrophilic area on the surface (Figure 6.13 C and F). The glycosylated 1.5 gen PAMAM hybrid resembles the glycosylated 3.5 gen PAMAM dendrimer (Figure 6.13 G).



Figure 6.14 Interpolated Charge Surfaces for: A - generation 2 triazine dendrimer modified with six glucosamine molecules, B - generation 2 triazine 0.5 PAMAM hybrid dendrimer modified with six glucosamine molecules; C - generation 2 triazine 1.5 PAMAM hybrid dendrimer modified with six glucosamine molecules; D - generation 3 triazine dendrimer modified with one glucosamine molecule; E - generation 3 triazine 0.5 PAMAM hybrid dendrimer modified with six glucosamine molecules; F - generation 3 triazine 1.5 PAMAM hybrid dendrimer modified with six glucosamine molecules; F - generation 3 triazine 1.5 PAMAM hybrid dendrimer modified with six glucosamine molecules; F - generation 3 triazine 1.5 PAMAM hybrid dendrimer modified with six glucosamine molecules; G - generation 3.5 PAMAM dendrimer modified with 8 glucosamine molecules. The red areas correspond to negatively charged residues and blue to positively charged surfaces.

Charge and its distribution, in the dendrimers surface, has also been identified as an important factor for interaction with the biological target. The interpolated charge surfaces were determined for all dendrimer involved in the study. The interpolated charge surfaces show that a similar compactness of the triazine and its 0.5 PAMAM hybrid (Figure 6.14 A, B, D and E), while the compactness of the 1.5 PAMAM hybrid is similar to that for the PAMAM (Figure 6.14 C, F and G). The hybrids with 1.5 gen PAMAM show more relaxed structures with fewer portions of the core exposed and a more varied charge distribution on their surface, showing the same trend as the findings described above.

Based on this analysis of the surface properties of the two hybrid dendrimers it was determined that the 0.5 gen PAMAM hybrid was more like the pure triazine dendrimer than the pure PAMAM dendrimer. Thus the 0.5 PAMAM hybrid would be expected to be inactive towards the TLR4 mediated inflammatory response. The analysis also showed that the glycosylated 1.5 gen PAMAM hybrid displayed comparable properties to the pure glycosylated PAMAM dendrimer, which is known as active and should be able to interact with biological target in a similar fashion having the same or comparable immuno-modulatory properties and therefore being capable of disrupting the TLR4 mediated inflammatory response.

To verify this hypothesis, the glycosylated generation 2 triazine hybrids, with 0.5 PAMAM (predicted inactive) and 1.5 PAMAM (predicted active) on their surface were synthesised and their biological relevance assessed. The results are shown for completeness.







TNF-α









Figure 6.15 Antagonist TLR4 induced inflammation activity of the generation 2 triazine 1.5 PAMAM hybrid dendrimer. It blocked LPS triggered pro-inflammatory chemokine (MIP-1 β and IL-8) and cytokine production (TNF- α and IL-6) but did not affect anti-inflammatory cytokines (IL-10 and IFN- β) production. The control was cultured primary human monocytes. * = p<0.001 when compared to the LPS positive control (Mann-Whitney U test).

The Real Time RT-PCR results show a clear decrease in the expression of the proinflammatory cytokines and chemokines (TNF- α , IL-6, MIP1- β and IL-8) when the generation 2 triazine 1.5 PAMAM hybrid dendrimer was added prior to the induction with LPS, when compared to the untreated cells, however it showed no effect on the anti-inflammatory cytokine IL-10. Due to the poor amplification efficiency of IFN- β it was difficult to observe a clear effect of the dendrimer conjugate. Nonetheless, these results reveal that the new hybrid molecule can act as an immuno-modulator and not immuno-suppressor of the TLR4-MD-2-LPS mediated pro-inflammatory responses (Figure 6.15). This is an extremely important feature as the immune system needs to produce small amounts of chemokines and cytokines as basic defence for the organism. However, this new hybrid was not able to reproduce the same levels inhibition as those observed for the glycosylated gen. 3.5 PAMAM dendrimers. The glycosylated hybrid presented a difference of one order of magnitude to the pure PAMAM, for most chemokines and cytokines and even two orders of magnitude in the case of TNF- α .







Figure 6.16 Real time RTPCR results for the generation 2 triazine 0.5 PAMAM hybrid dendrimer, which did not inhibit the expression of pro-inflammatory chemokine, MIP- 1β and cytokine, TNF- α , when their expression was induced with LPS.

The real time RT-PCR experiments with the generation 2 triazine 0.5 PAMAM, showed no difference in the levels of expression of the chemokines and cytokines tested, in pretreated cells, hence proving this hybrid is inactive towards the TLR4 mediated inflammatory response (Figure 6.16). One of the purposes of designing a new hybrid was to improve the chemical properties of the active molecules.



Figure 6.17 MALDI TOF analysis of 1 – glycosylated generation 3.5 PAMAM dendrimer; 2 – glycosylated generation 2 triazine 1.5 PAMAM hybrid dendrimer.

Mass spectrometry (Figure 6.17) and capillary electrophoresis (data not shown) revealed the glycosylated triazine generation 2 1.5 PAMAM hybrid presented an improvement on the level of polydispersity, however this molecule was not isolated as a single chemical entity. This heterogeneity is mainly due to the introduction of the PAMAM branches. The addition of PAMAM portions is achieved by Michael addition reactions which lead to incomplete processes and side reactions that cause the final product to be a mixture. The capillary electrophoresis results, performed by collaborators at Texas Christian University, indicated an 80% percent reduction of the number of species in the solution containing the glycosylated generation 2 triazine 1.5 PAMAM hybrid dendrimer (data not shown). This analysis also revealed an estimate loading of eight glucosamine molecules on the generation 2 triazine 1.5 PAMAM hybrid dendrimer. To validate the
molecular modelling techniques used and to confirm the accuracy of the properties predicted, a 2 ns molecular dynamics simulation was repeated and the properties for this hybrid with either 6 or 8 glucosamines determined. No significant difference in the properties determined was found. The average values for RMSD were 12 Å for the dendrimer with six glucosamine molecules and 11 Å for the dendrimer with eight glucosamine molecules. The surface area values determined were 8732 Å² and 8536 Å², respectively. Finally for the polar surface area was 5807 Å² for the dendrimer with six glucosamines and 5776 Å² for the dendrimer with eight glucosamine molecules.

As an overall, the biological evaluation of the glycosylated hybrid dendrimers showed that the predictions, that the glycosylated gen. 1.5 PAMAM hybrid displayed comparable properties to the gen. 3.5 PAMAM dendrimer and therefore should be capable of disrupting the TLR4 mediated inflammatory response, were correct but more importantly, that is possible by the observation of key features in active and inactive molecules to rationally design new molecules with desired biological activity towards defined targets.

Conclusion

Molecular modelling techniques were used to identify key surface properties of the known active glycolysed generation 3.5 PAMAM dendrimer and in a first instance compare these to the ones of known inactive generation two and three triazine dendrimers. The flexibility of these molecules, despite important for interaction with the biological target, was shown not to be the only factor important for the biological activity. Surface area and polar surface area were found to be important features for the desired activity. Finally and most importantly, the distribution of the hydrophobic and hydrophilic surfaces was found crucial for the interaction with MD-2 and thus inhibition of the LPS triggered inflammatory response. These findings were used in the rational design of dendrimer hybrids with varying number of generations of triazine as a core and PAMAM on their surface. It was deduced that the glycosylated 1.5 generation of PAMAM hybrid dendrimer should have similar surface properties as the active glycosylated PAMAM dendrimer and thus predicted active, whilst the glycosylated hybrids with 0.5 generation of PAMAM dendrimer were predicted inactive. These predictions were confirmed experimentally, through the synthesis and biological evaluation of the generation two glycosylated triazine/PAMAM hybrids.

Ultimately, this biology driven rational approach has allowed the design of new hybrid dendrimers with the desired biological activity, opening doors to the creation of new hybrid dendrimers that could act as agonists and antagonists for other important pathogen related TLR receptors.

CHAPTER VII – GENERAL CONCLUSIONS AND FUTURE WORK

General Conclusions

Shigellosis is a contagious infection that is a health problem. There is a large incidence in geographical areas of human conflict and shigellosis causes mortality especially in children³⁰. The antibiotics are used for therapy to eliminate the bacteria; however, there is no therapeutic approach to control the release of cytokines that cause tissue damage and sepsis. This inflammatory response is triggered by the LPS that is present in the bacterial membrane and is mediated by its interaction with the TLR4-MD-2 complex. Recent advances on how this complex recognises LPS and activates the cascade of events which culminates with the release of chemokines and cytokines have helped in the search for new therapies. Most approaches focus on the interactions with the hydrophobic pocket of the MD-2 protein where LPS is known to bind. The potential inhibitors contain a hydrophobic moiety that may potentially lead to problems with solubility and development of pharmaceutical formulations. The most successful example is Eritroran^{261, 262}, a synthetic lipid-A analogue which as of December 2009 is in Phase III clinical trials²⁶¹.

In a previous study that has been used as a basis for this PhD project, a different approach to mediate the reaction to LPS was taken. Glucosamine is a naturally occurring hydrophilic amino-saccharide with immuno-modulatory/anti-inflammatory effects²⁶³⁻²⁶⁶. However, concerns that it may induce some of the metabolic features of human diabetes when administrated in high doses have prevented its use in clinic²⁶⁷⁻²⁶⁹. It was found that glucosamine could be covalently conjugated to biocompatible PAMAM dendrimers as an efficient way to overcome the toxic effects of the high dosage required for unconjugated glucosamine. Consequently, highly water-soluble dendrimers with their hydrophilic surface modified with glucosamine were shown to inhibit the LPS triggered inflammatory response¹. The inhibition was achieved *via* a mechanism that appeared to rely on the polyvalency imparted by the dendrimer structure.

The glucosamine dendrimers that were found to have biological activity were derived from gen. 3.5 PAMAM dendrimers. These carboxylic acid terminated dendrimers present an anionic surface, which is known to be less cytotoxic and more biocompatible then positively charged dendrimers¹⁰¹⁻¹⁰³. Dendrimers conjugated with saccharides, or glycodendrimers, have been explored for biomedical applications^{76, 270, 271}. However, unlike the dendrimers used in this project, the conjugation is usually achieved via a linker or a spacer²⁷²⁻²⁷⁵ onto amine terminated dendrimers instead of carboxylic acid terminated dendrimers. While this strategy allows taking advantage of the low toxicity of the anionic surface of the dendrimers, the synthetic route does not allow for regiochemical control.

The resulting product is a mixture of glucosamine conjugates that cannot be fully characterised using analytical methods like NMR or mass spectrometry, which have been previously applied to dendrimers^{224, 276, 277}. In this project molecular modelling techniques were used to gain insights into the structural features of the gen. 3.5 PAMAM glucosamine conjugates and their interactions with the biological target (i.e. MD-2). With an increasing number of generations the hyper-branched structure becomes more complex, and different nomenclatures for these complex molecules have been proposed^{143, 278}. However, there are no reports of the use of these approaches to generate dendrimeric structures in a rapidly, versatile manner.

In this project an efficient method for 3-D structure generation of the PAMAM dendrimers was initially developed. This was necessary to provide a basis for the development of a general method to generate 3-D structures of hyper-branched molecules for molecular modelling analysis. Further simplification and automation of the method may aid the ease of use and compatibility with various software packages for simulation. This was a fundamental step for the study of the effect of glycosylation on the dendrimer structure and for the study of the interactions of the saccharide conjugate dendrimers with the LPS recognition system. It enabled the molecular dynamics simulations of glycosylated and non-modified PAMAM dendrimers with a new software package, Desmond, to be successfully used to conduct molecular dynamics simulations on these 3-D structures and to overcome the solvation problems that were initially encountered with NAMD.

A combination of methods using different levels of theory were then used to parameterise the unique environments present in these molecules. Although recent developments of the CHARMM force field included the general atom types and parameters for drug-like molecules²⁷⁹, the complexity of polymeric structures should justify the development of dedicated force field parameters. In the particular context of this project, this method was extremely important as it could be used to generate quite rapidly dendrimers with different surface patterns to help gain a better understanding of the biologically active mixture of dendrimers that were the subject of this thesis.

Molecular dynamics simulations using a range of different force fields (e.g. DREIDING, OPLS-AA, AMBER) have been previously used to understand behaviour of dendrimers^{99, 169-173}. Here, molecular dynamics simulations were initially used to select representative structures from 4.8 ns trajectories using dendrimers with increasing number of glucosamine molecules on their surface. This effort was focused on gaining a better understanding of the dynamic behaviour of the dendrimer conjugate as a function of glucosamine loading. Semi-empirical quantum chemistry methods were used to predict the electronic properties on significant portions of the dendrimers. Due to software limitations, it was not possible to calculate those properties for the whole dendrimer structure. However, it was expected that while the individual HOMO energy values of the whole dendrimer would differ from the HOMO values of the dendrimer segments, the HOMO-LUMO gaps would vary in the same way. This would lead to the same qualitative results.

Observation of HOMO-LUMO gaps on the molecules studied revealed that according to FMOT¹⁶² the more saccharide that is conjugated onto the surface, the less likely the next addition would take place. The electronic property studies revealed a range of possible loadings and distributions of saccharide conjugation on the dendrimer surface. This was consistent with the heterogeneity found in the experimental samples. However, the loading range could not be determined solely by electronic effects because the flexibility that is inherent in these molecules affects the loading and distribution of the glucosamine¹.

Detailed observation of the position of the terminal groups bearing the HOMO for each dendrimer was allied with the electronic properties. This indicated that when 3 glucosamine molecules are present on a quarter section of the dendrimer the terminal group surface where the HOMO is located led to reduced interaction with the reactants. This translates into a maximum loading of 12 glucosamine molecules per whole dendrimer. The HOMO energy values highly favour the addition of up to 8 glucosamine values per dendrimer. However, when these 8 molecules are present there is only a fifty percent chance the terminal group bearing the HOMO will be available. These results suggest an overall loading in the range of 8 to 12 glucosamine molecules bound to the carboxylate end groups on the dendrimer surface. This is in agreement with experimental data.

These studies also showed the impact of flexibility on the loading and distribution of the glucosamines and establishing dendrimer flexibility as an important component in their molecular properties. In fact, these gen 3.5 PAMAM dendrimers are highly flexible molecules as it was observed by monitoring their dynamic behaviour as demonstrated by their high RMSD values. In particular, the structure that was evaluated with ideal loading and distribution of glucosamine (i.e. eight glucosamine molecules conjugated to the surface) explored a larger conformational space then the dendrimers with seven or nine glucosamines, although all molecular properties of these three dendrimers were converging towards the end of simulations.

The observation of a dendrimer modified with one glucosamine in the presence and absence of the reagents, used in excess for its synthesis revealed that this excess does not affect the molecular properties of the dendrimers and that the presence of reagents could be excluded from further studies, decreasing the computational time of the computational experiments. The glycosylation of the dendrimers occurs in the presence of excess reagents, and these are known to remain in solution in trace levels. It is thought these trace impurities can be trapped within the dendrimer branches. Modeling revealed that this excess of reagents in solution does not have an impact on the dynamic behavior of glycosylated dendrimers. When assessing the dynamic behavior of known active PAMAM dendrimers, the range of saccharide loading determined was taken into account. Since the most favorable structure would be the dendrimer with eight evenly distributed glucosamine molecules, this loading plus/minus one was considered (e.g. 7-9). The study of the three molecules revealed that their dynamic behavior followed the

same profile, converging in most cases. The exception was the RMSD where the dendrimer with eight glucosamines was shown to explore a larger conformational space. The 8 glucosamine dendrimer was therefore selected for the interaction studies with the biological target.

In the assessment of the dynamic behaviour of gen. 3.5 PAMAMs, dendrimers without glucosamine, which are known to be inactive, and glucosamine conjugated dendrimers, which are known to be active, were compared. The key differences found between the active and the inactive PAMAM dendrimers were higher flexibility and a less compact structure for the PAMAM derivative that inhibited LPS. Flexibility is an important property of these molecules. Furthermore, it seems to be a major contributor to the loading and distribution of the glucosamine molecules on the surface and also having a role in the surface properties of the dendrimers' surface are believed to be important for the biological properties of the dendrimer conjugates. On the other hand, intramolecular hydrogen bonds may compromise the availability of the glucosamine molecules due to the folding in of the branches, were found to be present but did not show an impact on availability of glucosamine for intermolecular interactions.

At the beginning of this project a new method involving dialysis to purify the saccharide modified PAMAM dendrimers was developed and a new dendrimer conjugate was synthesised. Dialysis allowed a better purification of the PAMAM conjugates from carbodiimide derived coproducts. Since a divergent synthesis was used there was a mixture of conjugates that were produced. Characterisation techniques could only provide an estimate of saccharide loading, while exact conjugation site and distribution varied within the mixture.

A new dendrimer conjugate was synthesised, bearing 2-acetamido-2-deoxy-b-Dglucopyranosyl amine on its surface. This saccharide derivative is present on the *Shigella* LPS, which is known to trigger the TLR4 mediated inflammatory response. This new dendrimer conjugate displayed the same level of cytotoxicity and biological activity as the glucosamine conjugates. This new PAMAM conjugate did not bring much insight into the development of better and more potent dendrimer conjugates and its synthesis and purification were more complicated, so this line of research was abandoned. However, the fact that this saccharide did not alter the biological relevance of the conjugated dendrimers revealed information which proved to be a major determinant of the biological activity of the conjugated dendrimers. The study of this conjugate indicated the non-specific nature of the interaction of the PAMAM dendrimers with the biological target. The observation of the dynamic behavior of the glucosamine conjugated dendrimers also pointed in the same direction. No critical differences of the molecular properties were detected. There were only minor differences of the properties related with the surface on the molecules. These observations supported the hypothesis for the non-specificity of the interactions of the PAMAM conjugates with MD-2 protein.

The study of the interactions between the glucosamine dendrimer conjugates and the biological target was a complex task with many difficulties that demanded that some compromises be made. Dendrimers are hyperbranched molecules of large dimension that cannot be seen as small molecules nor as proteins. The macromolecular non-protein nature in the dendrimer structure caused problems in the application of molecular docking techniques for interaction studies. A survey of the docking programs available for academic researchers was undertaken. Over twenty different software packages (downloaded or accessed on servers). Only two were shown to be able to cope with the whole dendrimer structure. They are Hex and Patchdock. A common error for other protein-protein software included "residue not found", since this type of software builds the molecules structure based on the name of pre-defined residues. For small moleculeprotein interaction packages the common error arised due to the number of atoms on the molecule exceeding the maximum number of atoms the software could compute. With recent progress in the computational sciences and the formation of national grids, there is increasingly the possibility of computationally evaluating larger molecular systems longer periods of time. This means that the resources required to compute large systems that comprise polymeric structures have begun to be available and there is a need to develop software capable of recognising and dealing with these types of structures in terms of molecular docking.

Throughout these studies a strategy to examine the interaction between the glycosylated dendrimers and their biological target was a key goal. It was hypothesised

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that the interactions would be with the MD-2 protein. It was necessary to validate a combination of docking software was prior to use it to evaluate the possible interactions of the glycosylated denderimers and their biological target. The two crucial parameters that are important for docking are shape and electrostatic interaction. Their contributions were studied in depth. Hex and Patchdock were used for analysis of shape, complementarity between MD-2 and dendrimer, and the results were compared. Twenty representative structures of dendrimer without modifications (as a negative control) and the glycosylated dendrimer with eight glucosamines on the surface were docked against MD-2 as a target. For assessment of the electrostatic contribution an initial attempt to use a quarter section of the dendrimer endgroup surface and grow the remaining structure for dynamic assessment was done as had previously been described for a different dendrimer^{224, 276, 280}. However, GRID protocol was not able to deal with the number of atoms present. It was then decided not to use the smaller portions of the dendrimer with allowed number of atoms, since these could not be considered representative of the whole structure. Preliminary studies revealed that the use of the partial structures led to unrealistic solutions from which no conclusions could be taken. Nonetheless, this software allowed for the definition of "hot spots" for interactions between the TLR4-MD-2 complex and glucosamine which were later correlated with the shape results obtained from Hex and Patchdock.

The shape-based studies were consistent with the hypothesis of the dendrimer glucosamine conjugates interacting with MD-2 protein in the opening of the cavity which prevents LPS binding. The molecular dynamic simulations of the docking solutions further supported this mechanism for inhibiting LPS binding. These simulations studies revealed some important features of the interactions with MD-2 that can potentially lead to the development of better and more potent inhibitors. The presence of the dendrimer was shown to induce conformational changes on MD-2 that narrow the entrance of the cavity where LPS binds. Furthermore, it was possible to confirm the polyvalence nature of the dendrimer glucosamine interactions with MD-2 with the dendrimer changing conformation so that larger portions of the surface glucosamines could be involved in interacting with the protein.

There are still some aspects of the LPS recognition by MD-2 and TLR4 that have not been completely elucidated. Hence questions remain about the mechanism of interaction between LPS and MD-2 and TLR4. The order of events of LPS binding is still not resolved in the literature. Most results currently point in the direction of LPS binding to MD-2 prior to MD-2 binding to TLR4^{214, 215} but there is no absolute proof that the MD-2 is not bound to TLR4 at the time of LPS binding. Therefore, this possibility, though less likely, was also briefly inspected. Shape based docking with Hex was performed with the glycosylated dendrimers and the TLR4-MD-2 complex showing that the dendrimer could interact with both components of the complex with the potential to prevent dimerisation or to induce conformational changes on MD-2 to prevent LPS binding. However, this study was only preliminary and molecular dynamics simulations should be performed with these docking solutions. Due to the size and complexity of the dendrimer system, the use of molecular dynamics simulations would require large computational resource that is not currently available in these facilities at the moment. The possibility of using the National Grid Service (NGS) was considered however, Desmond was not available through the NGS and the dendrimer parameters needed to be further developed for the use with NAMD which is available via NGS.

The major differences found between the active and the inactive PAMAM dendrimers which might have an impact on their activity were larger conformational space explored and thus higher flexibility (RMSD calculations) and slightly less compact structure (gyration radius calculation). The total surface area and the polarity of the dendrimer surface are believed to be important in their biological role. Intramolecular hydrogen bonds of the dendrimers did not appear to have an impact on availability of glucosamine for intermolecular interactions.

The interaction studies of the glycosylated dendrimers with the MD-2 protein and the TLR4-MD-2 complex were performed using docking studies with different software packages indicated a possible mechanism for the interaction of the dendrimer glucosamine with the MD-2 accessory protein. The docking of unconjugated glucosamine molecules with the TRL4-MD-2 complex revealed a series of "hot-spots", allowing the detection of probable sites of interaction on the protein. The docking software was based on shape complementarity allowed the study of the whole dendrimer

with MD-2 was able to elucidate a possible mechanism which was consistent with the hypothesis that the glycosylated dendrimer interacts with charged residues in the opening of MD-2 pocket to block LPS binding. The dynamic behaviour of this interaction showed the dendrimer undergoes conformational changes so that more glucosamine molecules can move closer to MD-2. This appears to more efficiently block the MD-2 cavity. The presence of the dendrimer induces conformational changes on MD-2 causing the entrance of the pocket to become narrower. A preliminary study with the glycosylated dendrimer and the TLR4-MD-2 complex indicated that the dendrimer could interact with both components of the complex preventing dimerisation and/or causing conformational changes in MD-2 that would prevent LPS binding to the complex.

These studies have shown that it is possible to use molecular modelling techniques to gain insight into the structural features of dendrimeric structures and their interaction with biological targets. These studies opened doors to develop these techniques further so that similar systems can be evaluated in a time and cost effective manner. These molecular modelling techniques allowed the distinction between active and inactive molecules leading to the rational design of new hybrid molecules with tailored biological activity.

Molecular dynamics simulations performed with the gen. 3.5 PAMAM dendrimer modified with glucosamine (active) and with generations 2 and 3 triazine dendrimers modified with the same saccharide (inactive), revealed significant differences in the surface properties of these two molecules. It was hypothesised that these differences were key determinants of the biological variability of these compounds. The most dramatic differences were observed in the surface area values and the distribution of the hydrophilic and hydrophobic surfaces of the molecules. The compact structure of glycosylated triazine dendrimers allied with the exposed hydrophobic areas, when compared to the PAMAM dendrimer, were thought to be the main reason for their lack of biological activity.

It was then hypothesised that a hybrid dendrimer should maintain the same biological activity if it is able to reproduce the surface properties of the PAMAM dendrimer. It was thought this should be possible even if the core or a considerable proportion of the molecule was different than the structure near the endgroups. This concept was then applied to design a number of triazine-PAMAM hybrids with a different number of generations of the triazine core and surface PAMAM elements. The study revealed that a minimum of 1.5 generations PAMAM was necessary to reproduce the surface properties of the known active gen. 3.5 PAMAM glucosamine conjugate. For that reason hybrids with different cores but with 1.5 PAMAM generations were predicted to be active. Synthesis and biological evaluation of the hybrid molecules, performed by collaborators in Texas, confirmed the predictions and proved the concept of this method. These findings suggest that a rational design method can be developed and be expanded to other hyperbranched polymers so that better and more potent anti-inflammatory agents can be developed.

This work demonstrates how molecular modelling techniques can be used to gain some molecular insight into the structural features of dendrimeric structures and the binding interactions they may have with biological target. In this specific case, they allowed the proposal of possible mechanism of action for glycosylated PAMAM dendrimers. Furthermore, they allowed the establishment of a set of criteria to distinguish between active and inactive dendrimers and enabled the rational design of biological active hybrid molecules. As with most synthetic macromolecules there was considerable structural heterogeneity of the glucosamine dendrimers. The need to systematise and to develop a methodology for determining 3-D structure from the sequence of monomers used required considerable effort which may now be used for determining more defined structure property correlations which may inform future synthetic efforts.

Future Work

The work conducted for this PhD thesis has led to a few important conclusions and a mechanism of action of glycosylated dendrimer was proposed. However, there remains much that can still be elucidated. Several avenues of research were beyond the scope of this project in terms of computational resource available or time to conduct synthesis or biological experiment. More research could be pursued to design and predict properties of other polymers (linear or hyperbranched), especially if built from non-protein or non-DNA residues.

The basis of *in silico* method for 3D structure generation was established so it could be applied to other types of hyper-branched polymers. This can lead to development of a computational platform with the goal to automate coordinate generation and molecular property prediction for hyper-branched polymers and nanoparticles through the assembly of building blocks from a monomer sequence.

The application of hybrid molecular modelling methods to glycosylated PAMAM dendrimers, like QM/MM methods would be interesting for further analysis of the accessible surfaces of these molecules, especially since these methods would allow the study of the whole dendrimer. Furthermore, these methods may allow the prediction of the transition states and respective activation energies so more quantifiable criteria can be proposed for the reactivity and loading of moieties that modify the endgroups of the dendrimer. For example, methods such as SADDLE implemented in MOPAC can be used in conjunction with GROMACs for these purposes. Longer molecular dynamics simulations of the individual dendrimers would be of interest to repeat docking with the resulting representative structures for comparison and validation purposes of interaction studies. Since further insights about any dendrimer structure and conformational behaviour could be gained by using Principal Component Analysis (PCA) of the trajectories, an implementation of this method for Desmond trajectories could be of general interest for researchers in the field.

Further simulation studies of the complexes of the glycosylated dendrimers with MD-2 would be of great value for a more detailed understanding of the dynamic behaviour of these molecules and their interaction with the biological target. The longer simulations of the glycosylated dendrimer complexed with MD-2 might also bring more insight into specific residues on MD-2 opening relevant for interaction, which could then be tested by site directed mutagenesis techniques for a detailed mechanism of action. The same molecular dynamic simulations should be performed with the glycosylated dendrimers and the TLR4-MD-2 complex for a better understanding of the possible mechanism of action of the glycosylated dendrimers with the TLR4-MD-2 complex.

Finally, it would be interesting to expand the methods used to discriminate between experimentally determined active and inactive forms of dendrimers. The methods used to evaluate the triazine dendrimers and hybrids to other dendrimeric and hyperbranched molecules could be further refined and with greater computational resource used for the development of molecules with defined or more active immuno-modulatory properties, capable of disrupting the LPS triggered inflammatory response.

References

- 1. Shaunak, S., et al., *Polyvalent dendrimer glucosamine conjugates prevent scar tissue formation*. Nat Biotechnol, 2004. **22**(8): p. 977-84.
- 2. Takeda, K. and Akira, S., *Toll-like receptors in innate immunity*. Int. Immunol., 2005. **17**(1): p. 1-14.
- 3. Langman, R.E. and Cohn, M., A minimal model for the self-nonself discrimination: A return to the basics. Seminars in Immunology, 2000. 12(3): p. 189-195.
- 4. Borghesi, L. and Milcarek, C., Innate versus adaptive immunity: A paradigm past its prime? Cancer Res, 2007. 67(9): p. 3989-3993.
- 5. Beutler, B., *Innate immunity: An overview*. Molecular Immunology, 2004. **40**(12): p. 845-859.
- 6. Super, M. and Ezekowitz, R.A.B., *The role of mannose-binding proteins in host defense*. Infect Agent Dis 1992. 1(4): p. 194-199.
- 7. Matsushita, M. and Fujita, T., Activation of the classical complement pathway by mannose-binding protein in association with a novel cls-like serine protease. J. Exp. Med., 1992. **176**(6): p. 1497-1502.
- 8. Wright, S.D., et al., Cd14, a receptor for complexes of lipopolysaccharide (lps) and lps binding protein. Science, 1990. 249(4975): p. 1431-3.
- 9. Schumann, R.R., et al., Structure and function of lipopolysaccharide binding protein. Science, 1990. 249(4975): p. 1429-1431.
- 10. Hoffmann, M. and Dutton, R.W., *Immune response restoration with macrophage culture supernatants*. Science, 1971. **172**(3987): p. 1047-8.
- 11. Singh, P.K., et al., A component of innate immunity prevents bacterial biofilm development. Nature, 2002. 417(6888): p. 552-555.
- 12. Ganz, T. and Lehrer, R.I., Antimicrobial peptides of vertebrates. Curr Opin Immunol, 1998. 10(1): p. 41-44.
- 13. Beutler, B. and Hoffmann, J., *Innate immunity editorial overview*. Curr Opin Immunol, 2004. **16**(1): p. 1-3.
- 14. Hashimoto, C., Hudson, K.L., and Anderson, K.V., *The toll gene of drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein.* Cell, 1988. **52**(2): p. 269-279.
- 15. Rock, F.L., et al., *A family of human receptors structurally related to drosophila toll.* Proc Natl Acad Sci U S A, 1998. **95**(2): p. 588-593.
- 16. Takeuchi, O., et al., *Tlr6: A novel member of an expanding toll-like receptor family*. Gene, 1999. **231**(1-2): p. 59-65.
- 17. Means, T.K., Golenbock, D.T., and Fenton, M.J., *The biology of toll-like receptors*. Cytokine Growth F R, 2000. **11**(3): p. 219-232.
- 18. Jin, M.S. and Lee, J.O., Structures of the toll-like receptor family and its ligand complexes. Immunity, 2008. 29(2): p. 182-191.
- 19. Akira, S. and Hemmi, H., *Recognition of pathogen-associated molecular patterns by tlr family*. Immunol Lett, 2003. **85**(2): p. 85-95.
- 20. Gay, N.J. and Gangloff, M., Structure and function of toll receptors and their ligands. Annu Rev Biochem, 2007. 76: p. 141-165.

- 21. West, A.P., Koblansky, A.A., and Ghosh, S., *Recognition and signaling by toll-like receptors*. Annu Rev Cell Dev Bi, 2006. **22**: p. 409-437.
- 22. O'neill, L.A.J. and Bowie, A.G., *The family of five: Tir-domain-containing adaptors in toll-like receptor signalling.* Nature Reviews Immunology, 2007. 7(5): p. 353-364.
- 23. Matsushima, N., et al., Comparative sequence analysis of leucine-rich repeats (lrrs) within vertebrate toll-like receptors. BMC Genomics, 2007. 8(124).
- 24. Kobe, B. and Deisenhofer, J., *The leucine-rich repeat a versatile binding motif.* Trends Biochem Sci, 1994. **19**(10): p. 415-421.
- 25. Kajava, A.V., Structural diversity of leucine-rich repeat proteins. J Mol Biol, 1998. 277(3): p. 519-527.
- 26. Jin, M.S. and Lee, J.O., *Structures of tlr-ligand complexes*. Curr Opin Immunol, 2008. **20**(4): p. 414-419.
- 27. Shiga, K., Ueber den dysenterie bacillus (bacillus dysenteriae). Zentralblatt fur Bakteriol Parasitenkd Abt I Org., 1898. 24: p. 817-824.
- 28. Liu, B., et al., Structure and genetics of shigella o antigens. FEMS Microbiol Rev, 2008. 32(4): p. 627-53.
- 29. Sansonetti, P.J., Microbes and microbial toxins: Paradigms for microbialmucosal interactions - iii. Shigellosis: From symptoms to molecular pathogenesis. Am J Physiol-Gastr L, 2001. 280(3): p. 319-323.
- 30. Niyogi, S.K., *Shigellosis*. J Microbiol, 2005. **43**(2): p. 133-143.
- 31. Guessous, F., et al., Shiga toxin 2 and lipopolysaccharide induce human microvascular endothelial cells to release chemokines and factors that stimulate platelet function. Infection and Immunity, 2005. 73(12): p. 8306-8316.
- 32. Sansonetti, P.J. and Phalipon, A., *M cells as ports of entry for enteroinvasive pathogens: Mechanisms of interaction, consequences for the disease process.* Semin Immunol, 1999. **11**(3): p. 193-203.
- 33. Sansonetti, P.J., Genetic and molecular basis of epithelial cell invasion by shigella species. Rev Infect Dis, 1991. 13 Suppl 4: p. S285-92.
- 34. Zychlinsky, A. and Sansonetti, P.J., *Apoptosis as a proinflammatory event: What can we learn from bacteria-induced cell death?* Trends Microbiol, 1997. **5**(5): p. 201-4.
- 35. Struelens, M.J., et al., Shigella septicemia: Prevalence, presentation, risk factors, and outcome. J Infect Dis, 1985. 152(4): p. 784-90.
- 36. Bennish, M.L., *Potentially lethal complications of shigellosis*. Rev Infect Dis, 1991. **13 Suppl 4**: p. S319-24.
- 37. Raetz, C.R. and Whitfield, C., *Lipopolysaccharide endotoxins*. Annu Rev Biochem, 2002. **71**: p. 635-700.
- 38. Caroff, M. and Karibian, D., *Structure of bacterial lipopolysaccharides*. Carbohydr Res, 2003. **338**(23): p. 2431-2447.
- 39. Jeffrey D Esko, T.L.D., and Christian Rh Raetz Eubacteria and archaea, in *Essentials of glycobiology*, A. Varki, Editor. 2009, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y.
- 40. Holst, O., On the occurrence of d-glycero-d-mano-heptose in lipopolysaccharides. Polish Journal of Chemistry, 1999. 73(7): p. 1055-1067.

- 41. Vinogradov, E., et al., Structures of lipopolysaccharides from klebsiella pneumoniae. Eluicidation of the structure of the linkage region between core and polysaccharide o chain and identification of the residues at the non-reducing termini of the o chains. J Biol Chem, 2002. 277(28): p. 25070-81.
- 42. Vinogradov, E., Perry, M.B., and Conlan, J.W., *Structural analysis of francisella tularensis lipopolysaccharide*. Eur J Biochem, 2002. **269**(24): p. 6112-8.
- 43. Vinogradov, E.V., et al., *The core structure of the lipopolysaccharide from the causative agent of plague, yersinia pestis.* Carbohydr Res, 2002. **337**(9): p. 775-7.
- 44. Knirel' Iu, A. and Kochetkov, N.K., [structure of lipopolysaccharides from gram-negative bacteria. Iii. Structure of o-specific polysaccharides]. Biokhimiia, 1994. **59**(12): p. 1784-851.
- 45. Vinogradov, E., Structure of the o-specific polysaccharide chain of the lipopolysaccharide of bordetella hinzii. Carbohydr Res, 2002. **337**(10): p. 961-3.
- 46. Reeves, P.R., Variation in o-antigens, niche-specific selection and bacterial populations. FEMS Microbiol Lett, 1992. **79**(1-3): p. 509-16.
- 47. Reeves, P.P. and Wang, L., *Genomic organization of lps-specific loci*. Curr Top Microbiol Immunol, 2002. **264**(1): p. 109-35.
- 48. Pluschke, G., et al., Role of the capsule and the o antigen in resistance of o18:K1 escherichia coli to complement-mediated killing. Infect Immun, 1983. 42(3): p. 907-13.
- 49. Gemski, P., Jr., et al., Virulence of shigella flexneri hybrids expressing escherichia coli somatic antigens. Infect Immun, 1972. 6(2): p. 104-11.
- 50. Fahy, E., et al., A comprehensive classification system for lipids. J Lipid Res, 2005. 46(5): p. 839-61.
- 51. Shands, J.W., Jr. and Chun, P.W., *The dispersion of gram-negative lipopolysaccharide by deoxycholate. Subunit molecular weight.* J Biol Chem, 1980. **255**(3): p. 1221-6.
- 52. Makela, P.H., Jahkola, M., and Luderitz, O., A new gene cluster rfe concerned with the biosynthesis of salmonella lipopolysaccharide. J Gen Microbiol, 1970. **60**(1): p. 91-106.
- 53. Zahringer, U., Lindner, B., and Rietschel, E.T., Chemical structure of lipid a:Recent advances in structural analysis of biologycally active molecules, in Endotoxin in health and disease, H. Brade, et al., Editors. 1999, CRC Press.
- 54. Zahringer, U., et al., The lipopolysaccharide of legionella pneumophila serogroup 1 (strain philadelphia 1): Chemical structure and biological significance. Prog Clin Biol Res, 1995. **392**: p. 113-39.
- 55. Raetz, C.R.H., et al., *Lipid a modification systems in gram-negative bacteria*. Annu Rev Biochem, 2007. **76**: p. 295-329.
- 56. Beutler, B. and Cerami, A., *Tumor necrosis, cachexia, shock, and inflammation:* A common mediator. Annu Rev Biochem, 1988. **57**: p. 505-18.
- 57. Dinarello, C.A., Interleukin-1 and interleukin-1 antagonism. Blood, 1991. 77(8): p. 1627-52.
- 58. Li, A., et al., Comparison of the capacity of rhtnf-alpha and escherichia coli to induce procoagulant activity by baboon mononuclear cells in vivo and in vitro. Shock, 1996. 5(4): p. 274-9.

- 59. Drake, T.A., et al., Expression of tissue factor, thrombomodulin, and e-selectin in baboons with lethal escherichia coli sepsis. Am J Pathol, 1993. 142(5): p. 1458-70.
- 60. Medzhitov, R. and Janeway, C., Jr., *Innate immunity*. N Engl J Med, 2000. **343**(5): p. 338-44.
- 61. Van Duin, D., Medzhitov, R., and Shaw, A.C., *Triggering tlr signaling in vaccination*. Trends Immunol, 2006. 27(1): p. 49-55.
- 62. Russell, J.A., *Management of sepsis*. N Engl J Med, 2006. **355**(16): p. 1699-713.
- 63. Jerala, R., *Structural biology of the lps recognition*. Int J Med Microbiol, 2007. **297**(5): p. 353-363.
- 64. Miyake, K., *Endotoxin recognition molecules, toll-like receptor 4-md-2.* Semin Immunol, 2004. **16**(1): p. 11-6.
- 65. Tomalia, D.A., et al., *A new class of polymers starburst-dendritic macromolecules.* Polymer Journal, 1985. 17(1): p. 117-132.
- 66. Newkome, G.R., et al., *Micelles. Part 1. Cascade molecules: A new approach to micelles. A [27]-arborol.* The Journal of Organic Chemistry, 1985. **50**(11): p. 2003-2004.
- 67. Caminade, A.M., Laurent, R., and Majoral, J.P., *Characterization of dendrimers*. Adv Drug Deliv Rev, 2005. **57**(15): p. 2130-46.
- 68. Klajnert, B. and Bryszewska, M., *Dendrimers: Properties and applications*. Acta Biochimica Polonica, 2001. **48**(1): p. 199-208.
- 69. Hodge, P., Organic-chemistry polymer science branches out. Nature, 1993. 362(6415): p. 18-19.
- 70. Morefield, G.L., et al., Synthetic toll-like receptor 4 agonist enhances vaccine efficacy in an experimental model of toxic shock syndrome. Clinical and Vaccine Immunology, 2007. **14**(11): p. 1499-1504.
- 71. Hawker, C.J. and Frechet, J.M.J., *Preparation of polymers with controlled molecular architecture a new convergent approach to dendritic macromolecules*. J. Am. Chem. Soc., 1990. **112**(21): p. 7638-7647.
- 72. Cloninger, M.J., *Biological applications of dendrimers*. Curr Opin Chem Biol, 2002. 6(6): p. 742-748.
- 73. Mammen, M., Choi, S.K., and Whitesides, G.M., *Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors.* Angew Chem Int Edit, 1998. **37**(20): p. 2755-2794.
- 74. Sadler, K. and Tam, J.P., *Peptide dendrimers: Applications and synthesis.* J Biotechnol, 2002. **90**(3-4): p. 195-229.
- 75. Lee, C.C., et al., *Designing dendrimers for biological applications*. Nat Biotechnol, 2005. 23(12): p. 1517-26.
- 76. Niederhafner, P., et al., Glycopeptide dendrimers, part iii a review: Use of glycopeptide dendrimers in immunotherapy and diagnosis of cancer and viral diseases. J Pept Sci, 2008. 14(5): p. 556-587.
- 77. Boas, U. and Heegaard, P.M.H., *Dendrimers in drug research*. Chem Soc Rev, 2004. **33**(1): p. 43-63.
- 78. Svenson, S. and Tomalia, D.A., *Dendrimers in biomedical applications*reflections on the field. Adv Drug Deliv Rev, 2005. 57(15): p. 2106-29.

- 79. D'emanuele, A. and Attwood, D., *Dendrimer-drug interactions*. Adv Drug Deliv Rev, 2005. **57**(15): p. 2147-2162.
- 80. Van Hest, J.C.M., et al., *Polystyrene-dendrimer amphiphilic block copolymers* with a generation-dependent aggregation. Science, 1995. **268**(5217): p. 1592-1595.
- 81. Miklis, P., Çagin, T., and Goddard, W.A., *Dynamics of bengal rose encapsulated in the meijer dendrimer box.* J Am Chem Soc, 1997. **119**(32): p. 7458-7462.
- 82. Garcia-Bernabe, A., et al., Syntheses and phase-transfer properties of dendritic nanocarriers that contain perfluorinated shell structures. Chemistry, 2004. **10**(11): p. 2822-30.
- 83. Bosman, A.W., Janssen, H.M., and Meijer, E.W., *About dendrimers: Structure, physical properties, and applications.* Chem Rev, 1999. **99**(7): p. 1665-1688.
- 84. Haag, R., Supramolecular drug-delivery systems based on polymeric core-shell architectures. Angew Chem Int Ed Engl, 2004. **43**(3): p. 278-82.
- 85. Ihre, H.R., et al., Polyester dendritic systems for drug delivery applications: Design, synthesis, and characterization. Bioconjug Chem, 2002. 13(3): p. 443-52.
- 86. Frechet, J.M., *Dendrimers and supramolecular chemistry*. Proc Natl Acad Sci U S A, 2002. **99(8)**: p. 4782-7.
- 87. Esfand, R. and Tomalia, D.A., *Poly(amidoamine) (pamam) dendrimers: From biomimicry to drug delivery and biomedical applications.* Drug Discov Today, 2001. 6(8): p. 427-436.
- Venditto, V.J., Regino, C.A.S., and Brechbiel, M.W., Pamam dendrimer based macromolecules as improved contrast agents. Molecular Pharmaceutics, 2005. 2(4): p. 302-311.
- 89. Wolinsky, J.B. and Grinstaff, M.W., *Therapeutic and diagnostic applications of dendrimers for cancer treatment*. Adv Drug Deliv Rev, 2008. **60**(9): p. 1037-1055.
- 90. Morgan, M.T., et al., *Dendrimer-encapsulated camptothecins: Increased solubility, cellular uptake, and cellular retention affords enhanced anticancer activity in vitro.* Cancer Res, 2006. **66**(24): p. 11913-21.
- 91. Morgan, M.T., et al., *Dendritic molecular capsules for hydrophobic compounds*. J Am Chem Soc, 2003. **125**(50): p. 15485-9.
- 92. Wang, F., et al., Synthesis and evaluation of a star amphiphilic block copolymer from poly(epsilon-caprolactone) and poly(ethylene glycol) as a potential drug delivery carrier. Bioconjug Chem, 2005. **16**(2): p. 397-405.
- 93. Hong, S., et al., *The binding avidity of a nanoparticle-based multivalent targeted drug delivery platform.* Chem Biol, 2007. **14**(1): p. 107-15.
- 94. Patri, A.K., et al., Synthesis and in vitro testing of j591 antibody-dendrimer conjugates for targeted prostate cancer therapy. Bioconjug Chem, 2004. 15(6): p. 1174-81.
- 95. Bielinska, A., et al., Regulation of in vitro gene expression using antisense oligonucleotides or antisense expression plasmids transfected using starburst pamam dendrimers. Nucleic Acids Res, 1996. 24(11): p. 2176-82.

- 96. Bielinska, A.U., et al., Application of membrane-based dendrimer/DNA complexes for solid phase transfection in vitro and in vivo. Biomaterials, 2000. **21**(9): p. 877-87.
- 97. Kukowska-Latallo, J.F., et al., *Efficient transfer of genetic material into mammalian cells using starburst polyamidoamine dendrimers.* Proc Natl Acad Sci U S A, 1996. **93**(10): p. 4897-902.
- 98. Chen, C.Z. and Cooper, S.L., *Recent advances in antimicrobial dendrimers*. Advanced Materials, 2000. **12**(11): p. 843-846.
- 99. Von Der Lieth, C.-W., Frank, M., and Lindhorst, T.K., *Molecular dynamics* simulations of glycoclusters and glycodendrimers. Reviews in Molecular Biotechnology, 2002. **90**(3-4): p. 311-337.
- 100. Rojo, J. and Delgado, R., *Glycodendritic structures: Promising new antiviral drugs.* J Antimicrob Chemoth, 2004. **54**(3): p. 579-581.
- Roberts, J.C., Bhalgat, M.K., and Zera, R.T., Preliminary biological evaluation of polyamidoamine (pamam) starburst dendrimers. J Biomed Mater Res, 1996. 30(1): p. 53-65.
- Malik, N., et al., Dendrimers:: Relationship between structure and biocompatibility in vitro, and preliminary studies on the biodistribution of 125i-labelled polyamidoamine dendrimers in vivo. J Control Release, 2000. 65(1-2): p. 133-148.
- 103. Duncan, R. and Izzo, L., *Dendrimer biocompatibility and toxicity*. Adv Drug Deliv Rev, 2005. **57**(15): p. 2215-37.
- 104. Chen, H.T., et al., Cytotoxicity, hemolysis, and acute in vivo toxicity of dendrimers based on melamine, candidate vehicles for drug delivery. J Am Chem Soc, 2004. **126**(32): p. 10044-10048.
- 105. Wiwattanapatapee, R., et al., Anionic pamam dendrimers rapidly cross adult rat intestine in vitro: A potential oral delivery system? Pharmaceut Res, 2000. 17(8): p. 991-998-998.
- 106. Patri, A.K., Majoros, I.J., and Baker, J.R., *Dendritic polymer macromolecular carriers for drug delivery*. Curr Opin Chem Biol, 2002. **6**(4): p. 466-71.
- 107. Thomas, S.J., *The polyvalent properties of dendrimer based technology*, in *Department of Infectious Diseases Faculty of Medicine*. 2005, Imperial College London: London. p. 340.
- 108. Barril, X. and Soliva, R., Molecular modelling. Mol Biosyst, 2006. 2(12): p. 660-81.
- 109. Dorsett, H.W., A. . Overview of molecular modelling and ab initio molecular orbital methods suitable for use with energetic materials. 2000 [cited 2008; Available from: <u>http://www.dsto.defence.gov.au/publications</u>.
- 110. Gotwals, R.R.J.A.S., S. C. A chemistry educator's guide to molecular modeling; chapter7: Ab initio methods. 2007 [cited 2008; Available from: http://chemistry.ncssm.edu/book/.
- 111. Werner, H.-J.P.J.K. *Getting started with molpro*, *how an ab initio calculation works*. 2008 [cited 2008; Available from: www.molpro.net/info/current/doc/quickstart.

- 112. Stephens, P.J., et al., *Ab initio calculation of vibrational absorption and circular dichroism spectra using functional force fields*. J. Phys. Chem., 1994. **98**(45): p. 11623-11627.
- 113. Patey, M.D. and Dessent, C.E.H., A pw91 density functional study of conformational choice in 2-phenylethanol, n-butylbenzene, and their cations: Problems for density functional theory? The Journal of Physical Chemistry A, 2002. 106(18): p. 4623-4631.
- 114. Davidson, E.R. and Feller, D., Basis set selection for molecular calculations. Chem Rev, 1986. 86(4): p. 681-696.
- 115. Rassolov, V.A., et al., 6-31g* basis set for atoms k through zn. J Chem Phys, 1998. 109(4): p. 1223-1229.
- 116. Rassolov, V.A., et al., 6-31g* basis set for third-row atoms. J Comput Chem, 2001. 22(9): p. 976-984.
- 117. Mcnamara, J.P. and Hillier, I.H., Semi-empirical molecular orbital methods including dispersion corrections for the accurate prediction of the full range of intermolecular interactions in biomolecules. Phys Chem Chem Phys, 2007. 9(19): p. 2362-70.
- 118. Hoffmann, R., An extended h[u-umlaut]ckel theory. I. Hydrocarbons. J Chem Phys, 1963. **39**(6): p. 1397-1412.
- 119. Cramer, C.J., Semiempirical methods, in Essentials of computational chemistry: Theories and models. 2004, John Wiley & Sons Ltd: Chischester.
- 120. Pearson, R.G., *A simple model for vibrational force constants*. J Am Chem Soc, 1977. **99**(15): p. 4869-4875.
- 121. Dewar, M.J.S., et al., Development and use of quantum mechanical molecular models. 76. Am1: A new general purpose quantum mechanical molecular model. [erratum to document cited in ca103(2):11627f]. J Am Chem Soc, 1993. 115(12): p. 5348-5348.
- 122. Thomson, C., The molecular structures of 11-methyl and 1, 12dimethylbenz[a]anthracene: Purely theoretical semi-empirical am1 calculations are able to predict accurate structures of these polycyclic hydrocarbons. Carcinogenesis, 1989. 10(2): p. 317-320.
- 123. Stewart, J.J.P., *Optimization of parameters for semiempirical methods i. Method.* J Comput Chem, 1989. **10**(2): p. 209-220.
- 124. Stewartcomputationalchemistry. *Mopac2009*TM. 2009 [cited 2010; Available from: <u>http://openmopac.net/MOPAC2009brochure.pdf</u>.
- 125. Höltje, H.D.S., W.; Rognan, D.; Folkers, G., Molecular modeling: Basic principles and applications. 3 ed. 2008: Wiley-VCH.
- 126. Leach, A.R., *Molecular modelling principles and applications*. 2nd Edition ed. 2001, Harlow: Pearson Education Limited.
- Maginn, E.J. and Elliott, J.R., *Historical perspective and current outlook for* molecular dynamics as a chemical engineering tool. Ind Eng Chem Res. 49(7): p. 3059-3078.
- 128. Born, M. and Oppenheimer, R., Zur quantentheorie der molekeln. Annalen der Physik, 1927. **389**(20): p. 457-484.
- 129. Haynes, P.D., Linear-scaling methods in ab initio quantum-mechanical calculations, in Christ's College. 1998, University of Cambridge Cambridge.

- 130. Zielkiewicz, J., Structural properties of water: Comparison of the spc, spce, tip4p, and tip5p models of water. J Chem Phys, 2005. **123**(10): p. 104501.
- 131. Ercolessi, F. *A molecular dynamics primer*. 1997 [cited 2010; Available from: http://www3.ntu.edu.sg/home/mmhliang/molecular%20dynamics.html.
- 132. Ewald, P.P., Die berechnung optischer und elektrostatischer gitterpotentiale. Annalen der Physik, 1921. **369**(3): p. 253-287.
- 133. Darden, T., et al., New tricks for modelers from the crystallography toolkit: The particle mesh ewald algorithm and its use in nucleic acid simulations. Structure, 1999. 7(3): p. R55-R60.
- 134. Still, W.C., et al., Semianalytical treatment of solvation for molecular mechanics and dynamics. J Am Chem Soc, 2002. **112**(16): p. 6127-6129.
- 135. Lee, J.W., et al., Convergent synthesis of symmetrical and unsymmetrical pamam dendrimers. Macromolecules, 2006. **39**(6): p. 2418-2422.
- 136. Pearson, A.J. and Roush, W.R., *Activating agents and protecting groups*, New York: John Wiley & Sons, Inc.
- 137. Aiello, A.E., et al., *Persistent infection, inflammation, and functional impairment in older latinos.* J Gerontol A-Biol 2008. **63**(6): p. 610-618.
- 138. Dendritic Nanotechnologies, I. 2006 [cited 2010 03-08-2010]; Available from: http://dnanotech.com/dab.php.
- 139. Plumb, J.A., *Cell sensitivity assays: The mtt assay.* Methods Mol Med, 2004. **88**: p. 165-9.
- 140. Biologyonline. *Haemolysis*. 2008 [cited; Available from: <u>http://www.biology-online.org</u>.
- 141. Engel, R., Dendritic molecules: Concepts, syntheses, perspectives by george r. Newkome and charles n. Moorefield (university of south florida) and fritz vogtle (universitat bonn). Vch: Weinheim. 1996. Vi + 261 pp. Dm198. Isbn 3-527-29325-6. Journal of the American Chemical Society, 1997. 119(21): p. 5079-5079.
- 142. Lozac'h, N., Goodson, A.L., and Powell, W.H., *Nodal nomenclature-general principles*. Angew Chem Int Ed Engl, 1979. **18**(12): p. 887-899.
- 143. Roberts, B.P., et al., *The dotted cap notation: A concise notation for describing variegated dendrimers.* New J Chem, 2008. **32**(9): p. 1543-1554.
- 144. Newkome, G.R., et al., *A systematic nomenclature for cascade polymers*. J Polym Sci A Polym Chem, 1993. **31**(3): p. 641-651.
- 145. Friedhofen, J.H. and Vogtle, F., *Detailed nomenclature for dendritic molecules*. New J Chem, 2006. **30**(1): p. 32-43.
- 146. Lindahl, E., Hess, B., and Van Der Spoel, D., Gromacs 3.0: A package for molecular simulation and trajectory analysis. J Mol Model, 2001. 7(8): p. 306-317-317.
- 147. Brünger, A.T. *Xplor a system for x-ray crystallography and nmr* 1992 [cited 2010; Available from: <u>http://nmr.cit.nih.gov/xplor-nih/doc/current/xplor/</u>.
- 148. Charmmffparameters. [cited 2010; Available from: http://mackerell.umaryland.edu/CHARMM_ff_params.html.
- 149. Schrodingerinc. [cited 2010; Available from: www.schrodinger.com.

- 150. Jorgensen, W.L. and Tirado-Rives, J., Potential energy functions for atomiclevel simulations of water and organic and biomolecular systems. Proc Natl Acad Sci U S A, 2005. **102**(19): p. 6665-70.
- 151. Fariborz, M., et al., Macromodel an integrated software system for modeling organic and bioorganic molecules using molecular mechanics. J Comput Chem, 1990. 11(4): p. 440-467.
- 152. Phillips, J.C.Z., G; Kumar, S.; Kale, L. Namd: Biomolecular simulation on thousands of processors. 2002 [cited 2010; Available from: http://www.psc.edu/scaling_workshop/.
- 153. Humphrey, W., Dalke, A., and Schulten, K., Vmd: Visual molecular dynamics. J Mol Graphics, 1996. 14(1): p. 33-8.
- 154. The Open Babel Package, V. [cited 2010; Available from: http://openbabel.sourceforge.net/.
- 155. Powell, M.J.D., An efficient method for finding the minimum of a function of several variables without calculating derivatives. The Computer Journal, 1964. 7(2): p. 155-162.
- 156. Phillips, J.C., et al., Scalable molecular dynamics with namd. J Comput Chem, 2005. 26(16): p. 1781-1802.
- 157. Patel, S., Mackerell, A.D., and Brooks, C.L., Charmm fluctuating charge force field for proteins: Ii protein/solvent properties from molecular dynamics simulations using a nonadditive electrostatic model. J Comput Chem, 2004. 25(12): p. 1504-1514.
- 158. Mackerell, A.D., Jr., Banavali, N., and Foloppe, N., *Development and current status of the charmm force field for nucleic acids*. Biopolymers, 2000. **56**(4): p. 257-65.
- 159. Guvench, O., et al., Additive empirical force field for hexopyranose monosaccharides. J Comput Chem, 2008. 29(15): p. 2543-64.
- 160. Feller, S.E., et al., Molecular dynamics simulation of unsaturated lipid bilayers at low hydration: Parameterization and comparison with diffraction studies. Biophys J, 1997. 73(5): p. 2269-79.
- 161. Nobelprize.Org. [cited 2010; Available from: http://nobelprize.org/nobel_prizes/chemistry/laureates/1981.
- 162. Fukui, K., Role of frontier orbitals in chemical reactions. Science, 1982. 218(4574): p. 747-54.
- 163. Iupac, *Compendium of chemical terminology*, A.D.M.a.A. Wilkinson, Editor, Blackwell Scientific Publications: Oxford.
- 164. Fukui, K., *Recognition of stereochemical paths by orbital interaction*. Accounts Chem Res, 1971. **4**(2): p. 57-64.
- 165. Dannenberg, J.J., Using perturbation and frontier molecular orbital theory to predict diastereofacial selectivity. Chem Rev, 1999. **99**(5): p. 1225-42.
- 166. Wang, H., et al., A dft study of diels-alder reactions of o-quinone methides and various substituted ethenes: Selectivity and reaction mechanism. J Org Chem, 2005. 70(13): p. 4910-4917.
- 167. Chen, P. and Solomon, E.I., Frontier molecular orbital analysis of cun-o2 reactivity. J Inorg Biochem, 2002. 88(3-4): p. 368-374.

- 168. Tada, T., et al., *Molecular orbital interactions in the nanostar dendrimer*. J Phys Chem B, 2003. **107**(51): p. 14204-14210.
- 169. Suek, N.W. and Lamm, M.H., Effect of terminal group modification on the solution properties of dendrimers: A molecular dynamics simulation study. Macromolecules, 2006. **39**(12): p. 4247-4255.
- 170. Roberts, B.P., et al., Molecular dynamics of poly(l-lysine) dendrimers with naphthalene disulfonate caps. Macromolecules, 2009. 42(7): p. 2775-2783.
- 171. Lee, H., Baker, J.R., and Larson, R.G., Molecular dynamics studies of the size, shape, and internal structure of 0% and 90% acetylated fifth-generation polyamidoamine dendrimers in water and methanol. J Phys Chem B, 2006. **110**(9): p. 4014-4019.
- 172. Roberts, B.P., et al., *Molecular dynamics of variegated polyamide dendrimers*. Macromolecules, 2009. **42**(7): p. 2784-2794.
- 173. Lee, H. and Larson, R.G., Molecular dynamics study of the structure and interparticle interactions of polyethylene glycol-conjugated pamam dendrimers. J Phys Chem B, 2009. **113**(40): p. 13202-13207.
- 174. Vasumathi, V. and Maiti, P.K., Complexation of sirna with dendrimer: A molecular modeling approach. Macromolecules, 2010. **43**(19): p. 8264-8274.
- 175. Levy, R.M. and Gallicchio, E., Computer simulations with explicit solvent: Recent progress in the thermodynamic decomposition of free energies and in modeling electrostatic effects. Annu Rev Phys Chem, 2003. **49**(1): p. 531-567.
- 176. Pedretti, A., Villa, L., and Vistoli, G., Vega an open platform to develop chemo-bio-informatics applications, using plug-in architecture and script programming. J Comput Aid Mol Des, 2004. **18**(3): p. 167-173.
- 177. Stewart, J.J.P. *Mopac2009*TM. 2009 [cited 2010; Available from: <u>http://openmopac.net/MOPAC2009brochure.pdf</u>.
- 178. Maiorov, V.N. and Crippen, G.M., Significance of root-mean-square deviation in comparing three-dimensional structures of globular proteins. J Mol Biol, 1994. 235(2): p. 625-634.
- 179. Damm, K.L. and Carlson, H.A., Gaussian-weighted rmsd superposition of proteins: A structural comparison for flexible proteins and predicted protein structures. Biophys J, 2006. 90(12): p. 4558-4573.
- 180. Evangelos, A.C., Chaok, S., and Ken, A.D., Using quaternions to calculate rmsd. J Comput Chem, 2004. 25(15): p. 1849-1857.
- 181. Hajdin, C.E., et al., On the significance of an rna tertiary structure prediction. RNA. 16(7): p. 1340-1349.
- 182. Lobanov, M.Y., Bogatyreva, N.S., and Galzitskaya, O.V., *Radius of gyration as an indicator of protein structure compactness*. Mol Biol, 2008. **42**(4): p. 623-628.
- 183. Ohto, U. and Satow, Y., *Crystal twinning of human md-2 recognizing endotoxin cores of lipopolysaccharide*. J Synchrotron Radiat, 2008. **15**: p. 262-265.
- 184. Park, B.S., et al., *The structural basis of lipopolysaccharide recognition by the tlr4-md-2 complex*. Nature, 2009. **458**(7242): p. 1191-5.
- 185. Beamer, L.J., Carroll, S.F., and Eisenberg, D., *The bpi/lbp family of proteins: A structural analysis of conserved regions.* Protein Sci., 1998. 7(4): p. 906-914.

- Beamer, L.J., Carroll, S.F., and Eisenberg, D., Crystal structure of human bpi and two bound phospholipids at 2.4 angstrom resolution. Science, 1997. 276(5320): p. 1861-1864.
- 187. Lamping, N., et al., Effects of site-directed mutagenesis of basic residues (arg 94, lys 95, lys 99) of lipopolysaccharide (lps)-binding protein on binding and transfer of lps and subsequent immune cell activation. J Immunol, 1996. 157(10): p. 4648-4656.
- 188. Schumann, R.R., Lamping, N., and Hoess, A., Interchangeable endotoxinbinding domains in proteins with opposite lipopolysaccharide-dependent activities. J Immunol, 1997. **159**(11): p. 5599-605.
- 189. Kim, J.I., et al., Crystal structure of cd14 and its implications for lipopolysaccharide signaling. J Biol Chem, 2005. **280**(12): p. 11347-51.
- 190. Bell, J.K., et al., *The molecular structure of the toll-like receptor 3 ligandbinding domain.* Proc Natl Acad Sci U S A, 2005. **102**(31): p. 10976-80.
- 191. Gioannini, T.L., et al., Endotoxin-binding proteins modulate the susceptibility of bacterial endotoxin to deacylation by acyloxyacyl hydrolase. J Biol Chem, 2007. 282(11): p. 7877-84.
- 192. Juan, T.S., et al., Soluble cd14 truncated at amino acid 152 binds lipopolysaccharide (lps) and enables cellular response to lps. J Biol Chem, 1995. 270(3): p. 1382-7.
- 193. Muroi, M., Ohnishi, T., and Tanamoto, K.-I., Regions of the mouse cd14 molecule required for toll-like receptor 2- and 4-mediated activation of nf-kb. J Biol Chem, 2002. 277(44): p. 42372-42379.
- 194. Stelter, F., et al., Differential impact of substitution of amino acids 9-13 and 91-101 of human cd14 on soluble cd14-dependent activation of cells by lipopolysaccharide. J Immunol, 1999. 163(11): p. 6035-6044.
- 195. Pugin, J., et al., *Cd14 is a pattern recognition receptor*. Immunity, 1994. **1**(6): p. 509-16.
- 196. Dziarski, R., Recognition of bacterial peptidoglycan by the innate immune system. Cell Mol Life Sci, 2003. 60(9): p. 1793-1804.
- 197. Ohto, U., et al., Crystal structures of human md-2 and its complex with antiendotoxic lipid iva. Science, 2007. **316**(5831): p. 1632-1634.
- 198. Wright, S.D., Toll, a new piece in the puzzle of innate immunity. J Exp Med, 1999. 189(4): p. 605-609.
- 199. Shimazu, R., et al., *Md-2, a molecule that confers lipopolysaccharide responsiveness on toll-like receptor 4.* J Exp Med, 1999. **189**(11): p. 1777-1782.
- 200. Yang, H., et al., Cellular events mediated by lipopolysaccharide-stimulated tolllike receptor 4. Md-2 is required for activation of mitogen-activated protein kinases and elk-1. J Biol Chem, 2000. 275(27): p. 20861-6.
- 201. Akashi, S., et al., Human md-2 confers on mouse toll-like receptor 4 speciesspecific lipopolysaccharide recognition. Int Immunol, 2001. **13**(12): p. 1595-1599.
- 202. Viriyakosol, S., et al., *Md-2 binds to bacterial lipopolysaccharide*. J Biol Chem, 2001. **276**(41): p. 38044-51.
- 203. Mullen, G.E.D., et al., *The role of disulfide bonds in the assembly and function of md-2.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 3919-3924.

- 204. Schromm, A.B., et al., Molecular genetic analysis of an endotoxin nonresponder mutant cell line: A point mutation in a conserved region of md-2 abolishes endotoxin-induced signaling. J Exp Med, 2001. **194**(1): p. 79-88.
- 205. Kawasaki, K., Nogawa, H., and Nishijima, M., Identification of mouse md-2 residues important for forming the cell surface tlr4-md-2 complex recognized by anti-tlr4-md-2 antibodies, and for conferring lps and taxol responsiveness on mouse tlr4 by alanine-scanning mutagenesis. J Immunol, 2003. **170**(1): p. 413-20.
- 206. Visintin, A., et al., Lysines 128 and 132 enable lipopolysaccharide binding to md-2, leading to toll-like receptor-4 aggregation and signal transduction. J Biol Chem, 2003. 278(48): p. 48313-20.
- 207. Re, F. and Strominger, J.L., Separate functional domains of human md-2 mediate toll-like receptor 4-binding and lipopolysaccharide responsiveness. J Immunol, 2003. 171(10): p. 5272-5276.
- 208. Mancek, M., Pristovsek, P., and Jerala, R., *Identification of lps-binding peptide fragment of md-2, a toll-receptor accessory protein.* Biochem Biophys Res Commun, 2002. **292**(4): p. 880-5.
- 209. Hawkins, L.D., et al., A novel class of endotoxin receptor agonists with simplified structure, toll-like receptor 4-dependent immunostimulatory action, and adjuvant activity. J Pharmacol Exp Ther, 2002. 300(2): p. 655-61.
- 210. Saitoh, S., et al., Lipid a antagonist, lipid iva, is distinct from lipid a in interaction with toll-like receptor 4 (tlr4)-md-2 and ligand-induced tlr4 oligomerization. Int Immunol, 2004. 16(7): p. 961-9.
- 211. Visintin, A., et al., *Pharmacological inhibition of endotoxin responses is achieved by targeting the tlr4 coreceptor, md-2.* J Immunol, 2005. **175**(10): p. 6465-6472.
- 212. Meng, J., et al., Md-2 residues tyr 42, arg 69, asp 122 and leu 125 provide species specificity for lipid iva. J Biol Chem. 285(36): p. 27935-43.
- 213. Meng, J., Lien, E., and Golenbock, D.T., *Md-2-mediated ionic interactions* between lipid a and tlr4 are essential for receptor activation. J Biol Chem. **285**(12): p. 8695-702.
- 214. Akashi, S., et al., *Lipopolysaccharide interaction with cell surface toll-like receptor 4-md-2: Higher affinity than that with md-2 or cd14.* J Exp Med, 2003. **198**(7): p. 1035-1042.
- 215. Visintin, A., et al., *Md-2 expression is not required for cell surface targeting of toll-like receptor 4 (tlr4)*. J Leukoc Biol, 2006. **80**(6): p. 1584-92.
- 216. Connolly, M.L., Solvent-accessible surfaces of proteins and nucleic acids. Science, 1983. 221(4612): p. 709-13.
- 217. Connolly, M.L., Analytical molecular-surface calculation. J Appl Crystallogr, 1983. 16(Oct): p. 548-558.
- 218. Schneidman-Duhovny, D., et al., Patchdock and symmdock: Servers for rigid and symmetric docking. Nucleic Acids Res, 2005. 33(Web Server issue): p. W363-7.
- 219. Ritchie, D.W. and Kemp, G.J., Protein docking using spherical polar fourier correlations. Proteins, 2000. **39**(2): p. 178-194.

- 220. Macindoe, G., et al., *Hexserver: An fft-based protein docking server powered by graphics processors.* Nucl. Acids Res. **38**(suppl_2): p. W445-449.
- 221. Ritchie, D., Hex 5.0 user manual. 1996.
- 222. Goodford, P.J., A computational procedure for determining energetically favorable binding sites on biologically important macromolecules. J Med Chem, 1985. 28(7): p. 849-57.
- 223. Hampele, I.C., et al., Structure and function of the dihydropteroate synthase from staphylococcus aureus. J Mol Biol, 1997. **268**(1): p. 21-30.
- 224. Lalwani, S., et al., *Electrophoretic behavior of anionic triazine and pamam dendrimers: Methods for improving resolution and assessing purity using capillary electrophoresis.* Macromolecules, 2009. **42**(8): p. 3152-3161.
- 225. Chouai, A. and Simanek, E.E., Kilogram-scale synthesis of a second-generation dendrimer based on 1,3,5-triazine using green and industrially compatible methods with a single chromatographic step. J Org Chem, 2008. 73(6): p. 2357-2366.
- 226. Steffensen, M.B., et al., *Dendrimers based on [1,3,51-triazines.* J Polym Sci A Polym Chem, 2006. 44(11): p. 3411-3433.
- 227. Peter, T. and Leonard, J.P., Noncovalent synthesis of melaminecyanuric/barbituric acid derived nanostructures: Regio- and stereoselection. Eur J Org Chem, 2001. 2001(17): p. 3191-3205.
- 228. Quirke, J.M.E., Alan, R.K., and Charles, W.R., 1,3,5-triazines, in Comprehensive heterocyclic chemistry. 1984, Pergamon: Oxford. p. 457-530.
- 229. Whitesides, G.M., et al., Noncovalent synthesis using physical-organic chemistry to make aggregates. Accounts Chem. Res., 1995. 28(1): p. 37-44.
- 230. Steffensen, M.B. and Simanek, E.E., Synthesis and manipulation of orthogonally protected dendrimers: Building blocks for library synthesis. Angew Chem Int Edit, 2004. 43(39): p. 5178-5180.
- 231. Maciejewski, M., et al., *Guanamine dendrimerization*. J Macromol Sci Pure, 2000. **37**(7): p. 753-783.
- 232. Maciejewski, M., Concepts of trapping topologically by shell molecules. J Macromol Sci Chem, 1982. A17(4): p. 689-703.
- 233. Maciejewski, M., Topology in the chemistry of polymers. Polimery, 1995. 40(7-8): p. 404-409.
- 234. Christof, W. and Rolf, M., *Polynitrile- and polyamine-functional poly(trimethylene imine) dendrimers.* Angew Chem Int Edit, 1993. **32**(9): p. 1306-1308.
- 235. Zhang, W., et al., Orthogonal, convergent syntheses of dendrimers based on melamine with one or two unique surface sites for manipulation. J Am Chem Soc, 2001. **123**(37): p. 8914-8922.
- 236. Takagi, K., et al., *Triazine dendrimers by divergent and convergent methods*. J Polym Sci A Polym Chem, 2000. **38**(24): p. 4385-4395.
- 237. Takagi, K., et al., Synthesis of triazine dendrimers. Kobunshi Ronbunshu, 2000.
 57(10): p. 646-651.
- 238. Verheyde, B., Maes, W., and Dehaen, W., *The use of 1,3,5-triazines in dendrimer synthesis.* Mat Sci Eng C-Bio S, 2001. **18**(1-2): p. 243-245.

- 239. Verheyde, B. and Dehaen, W., Synthesis of dendrimers containing 1,3,4oxadiazoles. J Org Chem, 2001. 66(11): p. 4062-4064.
- 240. Gluzman Deceased., Y.L.O.U.S.R., Nj), Larocque, James P. (Highland Mills, Ny), O'hara, Bryan M. (Pearl River, Ny), Morin, John E. (Cold Spring, Ny), Ellestad, George A. (Pearl River, Ny), Mitsner, Boris (Nanuet, Ny), Ding, Wei-Dong (Nanuet, Ny), Raifeld, Yuri E. (Tarrytown, Ny), Nikitenko, Antonina A. (Tarrytown, Ny), *Triazine containing anionic compounds useful as antiviral agents*. 1998, American Cyanamid Company (Madison, NJ): United States.
- 241. Ding, W.-D., et al., Novel and specific respiratory syncytial virus inhibitors that target virus fusion. J Med Chem, 1998. **41**(15): p. 2671-2675.
- 242. Gazumyan, A., Mitsner, B., and Ellestad, G.A., Novel anti-rsv dianionic dendrimer-like compounds: Design, synthesis and biological evaluation. Curr Pharm Design, 2000. 6(5): p. 525-546.
- 243. Huntley, C.C., et al., *Rfi-641, a potent respiratory syncytial virus inhibitor.* Antimicrob. Agents Chemother., 2002. **46**(3): p. 841-847.
- 244. Maggon, K. and Barik, S., New drugs and treatment for respiratory syncytial virus. Rev Med Virol, 2004. 14(3): p. 149-168.
- 245. Lebreton, S., Newcombe, N., and Bradley, M., Antibacterial single-bead screening. Tetrahedron, 2003. 59(51): p. 10213-10222.
- 246. Neerman, M.F., et al., *In vitro and in vivo evaluation of a melamine dendrimer* as a vehicle for drug delivery. Int J Pharm, 2004. **281**(1-2): p. 129-132.
- 247. Venditto, V.J., et al., Intercepting the synthesis of triazine dendrimers with nucleophilic pharmacophores: A general strategy toward drug delivery vehicles. Chem Commun, 2009(37): p. 5541-5542.
- 248. Lim, J., et al., Design, synthesis, characterization, and biological evaluation of triazine dendrimers bearing paclitaxel using ester and ester/disulfide linkages. Bioconjugate Chemistry, 2009. 20(11): p. 2154-2161.
- 249. Lim, J.D. and Simanek, E.E., Synthesis of water-soluble dendrimers based on melamine bearing 16 paclitaxel groups. Org Lett, 2008. 10(2): p. 201-204.
- 250. Lo, S.-T., et al., Biological assessment of triazine dendrimer: Toxicological profiles, solution behavior, biodistribution, drug release and efficacy in a pegylated, paclitaxel construct. Mol Pharm: p. null-null.
- 251. Mintzer, M.A., et al., *Polycationic triazine-based dendrimers: Effect of peripheral groups on transfection efficiency.* New J Chem, 2009. **33**(9): p. 1918-1925.
- 252. Merkel, O.M., et al., Triazine dendrimers as nonviral gene delivery systems: Effects of molecular structure on biological activity. Bioconjugate Chem, 2009. 20(9): p. 1799-1806.
- Lai, L.L., et al., Star-shaped mesogens of triazine-based dendrons and dendrimers as unconventional columnar liquid crystals. J Org Chem, 2008. 73(2): p. 485-490.
- 254. Rothmann, M.M., et al., Donor-substituted 1,3,5-triazines as host materials for blue phosphorescent organic light-emitting diodes. Chem Mater. 22(7): p. 2403-2410.

- Brunet, P., Simard, M., and Wuest, J.D., Molecular tectonics. Porous hydrogenbonded networks with unprecedented structural integrity. J Am Chem Soc, 1997. 119(11): p. 2737-2738.
- 256. Fleet, G., et al., *Bis(1,3-dihydroxy-prop-2-yl)amine and derivatives thereof in the manufacture of polymers*, I.I. LIMITED, Editor. 2000: Oxford, GB.
- 257. [cited 2010; Available from: <u>http://avogadro.openmolecules.net</u>.
- 258. Halgren, T.A., Merck molecular force field .1. Basis, form, scope, parameterization, and performance of mmff94. J Comput Chem, 1996. 17(5-6): p. 490-519.
- 259. Hilal, S.H., Karickhoff, S.W., and Carreira, L.A., A rigorous test for sparc's chemical reactivity models: Estimation of more than 4300 ionization pkas. Quant Struct-Act Rel, 1995. 14(4): p. 348-355.
- 260. Zloh, M., et al., Identification and insertion of 3-carbon bridges in protein disulfide bonds: A computational approach. Nat Protoc, 2007. 2(5): p. 1070-83.
- 261. A controlled comparison of eritoran tetrasodium and placebo in patients with severe sepsis. February 2010 [cited 2010; Available from: http://www.clinicaltrials.gov/show/NCT00334828.
- 262. Kim, H.M., et al., Crystal structure of the tlr4-md-2 complex with bound endotoxin antagonist eritoran. Cell, 2007. 130(5): p. 906-917.
- 263. Fukasawa, M., et al., Modulation of proline and glucosamine incorporation into tissue repair cells by peritoneal macrophages. J Surg Res, 1989. 46(2): p. 166-71.
- 264. Lehto, M. and Jarvinen, M., Collagen and glycosaminoglycan synthesis of injured gastrocnemius muscle in rat. Eur Surg Res, 1985. 17(3): p. 179-85.
- 265. Hua, J., Sakamoto, K., and Nagaoka, I., Inhibitory actions of glucosamine, a therapeutic agent for osteoarthritis, on the functions of neutrophils. J Leukoc Biol, 2002. 71(4): p. 632-40.
- 266. Meininger, C.J., et al., *Glucosamine inhibits inducible nitric oxide synthesis*. Biochem Biophys Res Commun, 2000. **279**(1): p. 234-9.
- 267. Marshall, S., Bacote, V., and Traxinger, R.R., Discovery of a metabolic pathway mediating glucose-induced desensitization of the glucose transport system. Role of hexosamine biosynthesis in the induction of insulin resistance. J Biol Chem, 1991. 266(8): p. 4706-12.
- 268. Han, D.H., Chen, M.M., and Holloszy, J.O., *Glucosamine and glucose induce insulin resistance by different mechanisms in rat skeletal muscle*. Am J Physiol Endocrinol Metab, 2003. **285**(6): p. E1267-72.
- Shepherd, P.R. and Kahn, B.B., Glucose transporters and insulin actionimplications for insulin resistance and diabetes mellitus. N Engl J Med, 1999.
 341(4): p. 248-57.
- 270. Niederhafner, P., Sebestik, J., and Jezek, J., *Glycopeptide dendrimers. Part i.* J Pept Sci, 2008. 14(1): p. 2-43.
- 271. Niederhafner, P., Sebestik, J., and Jezek, J., *Glycopeptide dendrimers. Part ii.* J Pept Sci, 2008. 14(1): p. 44-65.
- 272. Boysen, M.M.K. and Lindhorst, T.K., ['Jsugaring' carbosilane dendrimers via hydrosilylation. Tetrahedron, 2003. 59(22): p. 3895-3898.

- 273. Ashton, P.R., et al., Synthesis of glycodendrimers by modification of poly(propylene imine) dendrimers. Chemistry A European Journal, 1997. 3(6): p. 974-984.
- 274. Zanini, D. and Roy, R., Practical synthesis of starburst pamam αthiosialodendrimers for probing multivalent carbohydrate-lectin binding properties. J Org Chem, 1998. 63(10): p. 3486-3491.
- 275. Kieburg, C. and Lindhorst, T.K., *Glycodendrimer synthesis without using protecting groups*. Tetrahedron Letters, 1997. **38**(22): p. 3885-3888.
- 276. Lalwani, S., et al., *Mimicking pamam dendrimers with amphoteric, hybrid triazine dendrimers: A comparison of dispersity and stability.* Macromolecules, 2009. **42**(17): p. 6723-6732.
- 277. Giordanengo, R., et al., Propagation of structural deviations of poly(amidoamine) fan-shape dendrimers (generations 0-3) characterized by maldi and electrospray mass spectrometry. Int J Mass Spectrom, 2007. 266(1-3): p. 62-75.
- 278. Mendenhall, G.D., *Fractal notation for nearly-symmetrical dendrimers*. J Polym Sci A Polym Chem, 1998. **36**(16): p. 2979- 2983.
- 279. Vanommeslaeghe, K., et al., Charmm general force field: A force field for druglike molecules compatible with the charmm all-atom additive biological force fields. J Comput Chem, 2010. **31**(4): p. 671-690.
- 280. Ivanov, A.A. and Jacobson, K.A., *Molecular modeling of a pamam-cgs21680* dendrimer bound to an a2a adenosine receptor homodimer. Bioorg Med Chem Lett, 2008. **18**(15): p. 4312-4315.

APPENDIXES

2.1 NMR and MALDI analysis of the reagents involved in the synthesis of generation 3.5 dendrimer glucosamine

a) EDC

The MALDI analysis was performed in the same mass range as dendrimer glucosamine to determine if the impurities observed had their source in this reagent.



Figure A2.1.1 MALDI-TOF analysis of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, the cross linking agent used in the synthesis of generation 3.5 dendrimer glucosamine.

Observing the spectrum it is possible to see that there are no impurities in the region under study.

The results of the NMR analysis of this compound are displayed below.

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Figure A2.1.2 ¹H-NMR analysis of 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide, the cross linking agent used in the synthesis of generation 3.5 dendrimer glucosamine.

Looking at the spectrum it is possible to identify a triplet around 1 ppm indicating a CH_3 group just next to a CH_2 (a) and a quadruplet close to 3 ppm showing the presence of a CH_2 group next to a CH_3 (b), using the 1 ppm peak as reference for integration it possible to identify one other peak close to 3.3 ppm corresponding to 6 H, this peak was assigned as corresponding to the 2 Me groups (f,g), the reason why they are shifted down field is its proximity to the N.



Figure A2.1.3 2D COSY analysis of 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide, the cross linking agent used in the synthesis of generation 3.5 dendrimer glucosamine.

COSY NMR allows the visualisation of hydrogens that are three bonds way from each other. Observing the spectrum it is possible to see a correlation peak around 3 ppm corresponding to Carbons assigned and a and b, previously identified in the ¹H-NMR spectrum. On the 1HNMR projection, around 2 ppm there is a singlet presenting two correlation peaks, this means this CH₂ group is in the middle of two other carbons. Looking at the structure there is only one carbon in this circumstances and it was assigned with the letter d.



Figure A2.1.4 2D NOESY analysis of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, the cross linking agent used in the synthesis of generation 3.5 dendrimer glucosamine.

2D Noesy NMR allows the visualization of any two hydrogens close enough to interact. Looking at the correlation peaks of the unidentified peaks with the identified ones it is possible to distinguish between the two of them:

- One correlating to the 2Me groups (e, 3.8ppm);
- One that correlates only with the middle CH_2 (c, 3.4ppm).

b) Glucosamine Hydrochloride

The MALDI analysis was performed in the same mass range as dendrimer glucosamine to determine if the impurities observed had their source in this reagent.



Figure A2.1.5 MALDI-TOF analysis of Glucosamine hydrochloride used in the synthesis of generation 3.5 dendrimer glucosamine.

Observing the spectrum it is possible to see that there are no impurities in the region under study.

The results of the NMR analysis of this compound are displayed below.



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Figure A2.1.6 ¹H-NMR analysis of Glucosamine hydroxide used in the synthesis of generation 3.5 dendrimer glucosamine.

The ¹H-NMR spectrum of glucosamine hydrochloride shows more peaks then what would be expected indicating the presence of stereoisomers. Around 5.4 ppm it is possible to observe a peak corresponding to the anomeric proton (H1).


Figure A2.1.7 2D TOCSY analysis of Glucosamine hydroxide used in the synthesis of generation 3.5 dendrimer glucosamine, each colour represents one stereoisomer.

Looking at the 2D TOCSY spectrum for this compound it is clear the present of the two isomers of glucosamine hydroxide present in the sample. The presence of isomers leads to peak overlap making the accurate analysis of the spectrum difficult. Based on the correlation peaks observed the following assignments were done:

Isomer 1 (red) – 2.9 ppm C2 - 3.4 ppm C4 - 3.6 ppm C3 Isomer 2 (blue) – 3.2 ppm C2 - 3.4 ppm C4 - 3.8 ppm C3

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Figure A2.1.8 2D HMQC analysis of Glucosamine hydroxide used in the synthesis of generation 3.5 dendrimer glucosamine.

HMQC shows correlation peaks between a carbon and hydrogen directly bound to it. Observing the HMQC spectrum for glucosamine hydroxide it was possible to estimate the peaks corresponding to C5 (\sim 70 ppm) and C6 (\sim 60 ppm), however, it was not possible to distinguish the isomers.

According to the supplier (Sigma-Aldrich, UK) the glucosamine isomers differ by the stereochemistry at C1 position.



c) PAMAM dendrimer 1, 4-diaminobutane core generation 3.5

Figure A2.1.9 MALDI-TOF analysis of PAMAM dendrimer 1, 4-diaminobutane core generation 3.5, used in the synthesis of generation 3.5 dendrimer glucosamine.

Looking at the spectrum B it is possible to see a broad peak representing the dendrimer with different Na loadings. The presence of sodium ions greatly reduces the sensitivity

of the method leading to broadness of peaks. In spectrum A it is possible to observe some impurities found in the purchased dendrimer, also because of the presence of salt the these are not very clear.



Figure A2.1.10 ¹H-NMR analysis of the PAMAM dendrimer 1, 4-diaminobutane core generation 3.5, used in the synthesis of generation 3.5 dendrimer glucosamine.

This NMR analysis was performed in an attempt to understand the origin of the impurities found in the synthesised dendrimer glucosamine. In this perspective no assignments were made since NMR of PAMAM dendrimer has previously been assigned. Looking at the spectrum it is possible to see the presence of small impurities around 6ppm.

2.2 Calculation of the aminosaccharide loading of the gen. 3.5 PAMAM dendrimers using areas under the peaks obtained from NMR spectral data¹

	DG	DGS
Integral value for signal(s) at/between:		
5.23 ppm & 4.75 ppm (1H aminosaccharide, A)	1.00 + 0.89 = 1.89	1.00 + 0.64 = 1.64
4.4 to 2.4 ppm (6H aminosaccharide + 12H urea + 740H den., B)	175.33	147.93
1.3 to 1.0 ppm (3H urea, C)	2.05	0.92
Calculations:		
Integral for 1H dendrimer (D) ([B - (6 A + 4 C)]/740)	0.21	0.18
Mol ratio of aminosaccharide to dendrimer (E) (A/D)	8.98	9.03
% loading of aminosaccharide (E/64 x 100%)	14	14

3.1 Alternative core blocks

DCB – Dodecane Core Block



DBA – Dendrimer Block A

H₂N NH₂

3.2 Topology File

Please refer to CD attached

3.3 Parameter File

Please refer to CD attached

3.4 Generate.inp file

Please refer to CD attached

3.5 Random.inp file

Please refer to CD attached

3.6 Refine.inp file

Please refer to CD attached

4.1 PatchDock Parameters

PatchDock Parameter File for md2.pdb-d8s_1.pdb

receptorPdb md2.pdb ligandPdb d8s 1.pdb receptorMs md2.pdb.ms ligandMs d8s_1.pdb.ms #receptorActiveSite site1.txt #ligandActiveSite site2.txt protLib /usr/local/modelling/PatchDock/chem.lib log-file patch_dock.log log-level 2 # Distance constraint parameters: distanceConstraints <rec atom index> <lig atom index> <dist thr> # # <rec atom index> - receptor atom used for constraint # <lig atom index> - ligand atom used for constraint # <dist thr> - maximum allowed distance between the specified atom centers #distanceConstraints rec atom index lig atom index dist thr #distanceConstraintsFile file name # Surface Segmentation Parameters: # receptorSeg <low patch thr> <high patch thr> <prune thr> <knob> <flat> <hole> # # <hot spot filter type> # v patch thr>,<high patch thr> - min and max patch diameter # <prune thr> - minimal distance between points inside the patch # <knob> <flat> <hole> - types of patches to dock (1-use, 0-do not use) # <hot spot filter type> :None - 0, Antibody - 1, Antigen - 2 # Protease - 3, Inhibitor - 4, Drug - 5 receptorSeg 10.0 20.0 1.5 1 0 1 0 ligandSeg 10.0 20.0 1.5 1 0 1 0 # Scoring Parameters: scoreParams <small_interfaces_ratio> <max_penetration> <ns_thr> # <rec as thr> as thr> <patch res num> <w1 w2 w3 w4 w5> # # <small_interfaces_ratio> - the ratio of the low scoring transforms to be removed # <max_penetration> - maximal allowed penetration between molecules surfaces # <ns thr> - normal score threshold # <rec_as_thr> <lig_as_thr> - the minimal ratio of the active site area in the solutions # <patch res_num> - number of results to consider in each patch # <wl w2 w3 w4 w5> - scoring weights for ranges: [-5.0,-3.6],[-3.6,-2.2],[-2.2,-1.0],[-1.0,1.0],[1.0-up] #

scoreParams 0.3 -2.0 0.5 0.0 0.0 1500 -8 -4 0 1 0

- # Desolvation Scoring Parameters:
- # desolvationParams <energy thr> <cut off ratio>
- # <energy_thr> remove all results with desolvation energy higher thanthreshold <cut off ratio> - the ratio of low energy results to be kept
- # First filtering with energy_thr is applied and the remaining results
- # can be further filtered with cut_off_ratio.

desolvationParams 500.0 1.0

Grid Parameters:

receptorGrid <gridStep> <maxDistInDistFunction> <vol_func_radius>
NOTE: the vol_func_radius of small molecules and peptides should be 3A!
receptorGrid 0.5 6.0 6.0
ligandGrid 0.5 6.0 6.0

Energy Parameters: vdWTermType 1 attrVdWEnergyTermWeight 1.01 repVdWEnergyTermWeight 0.5 HBEnergyTermWeight 1.0 ACE_EnergyTermWeight 1.0 piStackEnergyTermWeight 0.0 confProbEnergyTermWeight 0.1 COM_distanceTermWeight 1.07 energyDistCutoff 6.0 elecEnergyTermWeight 0.1 radiiScaling 0.8

4.2 Rebol Script

REBOL [Title: "PROTEIN-LIGAND DISTANCES" Date: 14-MAY-2008; 06-MARCH-2010 Author: "RADEK WOJCIK, TERESA BARATA & MIRE ZLOH" Version: 1.0.0 Usage: { This script measures distances between atoms of selected residues of protein and all atoms of ligand. }] ; Load the VEGA interface

; Load the VEGA interface do %..\Scripts\Common\Vega.r ; Open the comunication port

VegaOpen VegaDefHost VegaDefPort VegaDefUser VegaDefPass VegaCmd {CONCLS} VEGACMD {CONSET 20000 50}

; Check if a calculation is already running VegaCmd "PluginGet IsRunning" if equal? VegaRes "1" [VegaCmd rejoin[{MessageBox "Calculation already running" "ERROR" 16}] VegaClose quit 1 ; Change the mouse cursor VegaCmd {Cursor Busy} ; Print into the VEGA console VegaCmd {Text "" 1} VegaCmd {Text "**** Interatomic distances ****" 1} VegaCmd {Text "" 1} ; Clear all molecules VegaCmd {New} ; Get the VEGA installation directory VegaCmd {Get VegaDir} MolPath: join VegaRes "Molecules\" ; Get the number of atoms VegaCmd {Get TotAtm} TotAtm: to-integer VegaRes ResSeq: "ResSeq" change-dir to-rebol-file "C:\Documents and Settings\teresa barata\My Documents\Teresa Wind\vegaMD\" current-dir: to-local-file what-dir current-dir: "C:\Documents and Settings\teresa barata\My Documents\Teresa Wind\vegaMD\D8S1\" current-files: read to-rebol-file current-dir foreach file current-files [VegaCmd {New} if find file ".pdb" [VegaCmd rejoin [{Open "} current-dir file {"}] VegaCmd "Get TotAtm" TotLig: to-integer VegaRes VegaCmd {mColorByMol} VegaCmd rejoin [{Text "Ligand filename: } :file {" 1}] VegaCmd rejoin [{Text "No. of ligand's atoms: } TotLig {" 1}] VegaCmd {mNormalize} VegaCmd {mColorByMol} VegaCmd {Get TotAtm} Total: to-integer VegaRes VegaCmd rejoin[{Text "No. of all atoms: } Total {" 1}] Totprot: 2317 Totlig: 1856 ;VegaCmd {mPickRemMon}

;Measure distances

```
max: to-decimal 3
          for i 2875 Total 1[
 CheckRes: "false"
   for j 1 Totprot 1 [
          VegaCmd rejoin ["Distance " i " " j " 0"]
          distA: to-decimal VegaRes
             if CheckRes = "false" [
              if distA < max [
   VegaCmd rejoin [ "AtmGet " j " ResSeq" ]
     ProtNum: to-integer VegaRes
    VegaCmd rejoin ["AtmGet " i " ResSeq" ]
;
      LigNum: to-integer VegaRes
;
            VegaCmd rejoin [{Text "Distance, } ProtNum {,} i - 2317 {,} distA {" 1}]
             VegaCmd rejoin [{Text "CLOSE TO RESIDUE NUMBER: } ProtNum { "1}]
;
             CheckRes: "true"
                                  1
            ]; End for if statement checking the residue distances
         ]; End for (j)
]; End for (i)
VegaCmd {CONSAVE "C:\Documents and
Settings\teresa_barata\MyDocuments\Teresa_Wind\vegaMD\d8sI.txt"}
]; End for (files)
; Change the mouse cursor to default
```

VegaCmd {Cursor Default}

; Close the comunication port

| VegaClose

5.1 Docking studies of maltose and sucrose with the MD-2 protein, using GRID

Method

Greater (Molecular Discovery Ltd) was used to generate the ".kout" files. Glue (Molecular Discovery Ltd) was used for docking. This requires either ".kout" or "mol2" files of ligands used in the docking process. So "pdb" files were converted to .kout. For the docking process, all available probes were selected to generate molecular interactions fields (MIFs). A maximum number of 100 binding sites were used with an energy cut off of -100 Kcal/mol. The maximum iterations value was set to 120. For ligand flexibility 5 rotatable bonds were allowed and the electrostatic term was included for interaction energies calculation. Maltose and sucrose were docked against MD-2 as in the crystal structure (2E59).

Results

The binding sites of these negative control molecules are clearly not interacting with the opening of the MD-2, developing interactions with the back and top of MD-2.



Maltose

Sucrose

Figure A5.1.1 MD-2 binding sites for maltose and sucrose, obtained with GRID. MD-2 is represented as ribbons, the saccharides are represented as stick and the lowest energy structure coloured red.

5.2 Molecular dynamics simulation of dendrimer glucosamine and MD-2 protein, where no glucosamine were initially close to the protein

Method

The "pdb" file with the docking solution was loaded into Maestro and Desmond was used to perform molecular dynamics simulation with explicit solvent. The system was built using SPC solvation model and the size of the box determined automatically by creating a 10Å buffer around the glycosylated dendrimer and MD-2 protein. The molecular dynamics simulation was performed for 1 ns at 300 K and 1.03 bar, including structure minimisation and relaxation steps. Snapshot structures were recorded every 4.8 ps.

Results

In this simulation the glucosamine molecules on the dendrimer surface were initially far from the MD-2 protein (Figure A5.2.1 A). As the simulation progresses it is possible to observe that the dendrimer moves away from MD-2, due to the repulsion of the negative charges on the dendrimer branches (Figure A5.2.1 B), rearranging itself so that the glucosamine molecules can become closer to MD-2 protein, which is observed in the end of the simulation (Figure A5.2.1 C).



Figure A5.2.1 Three structures representing the snapshots of molecular dynamics simulation of the glycosylated dendrimer with MD-2. A - 0 ns; B - 400 ps and C - 1 ns. MD-2 is displayed as ribbons, the glycosylated dendrimer as tube and the glucosamine molecules on the surface of the dendrimer as CPK.

6.1 Manuscript of the submitted paper

Please refer to the CD attached

6.2 Comparison between Implicit and Explicit solvation simulation of a generation 3.5 PAMAM dendrimer modified with glucosamine

Methods

The structures used were both generated as described in Chapter III, however the initial structure for each simulation was not the same.

Implicit solvation simulation was performed at 300k by Macromodel using OPLS2005 force field (Jorgensen, 2205) usingGeneralized Born/ Surface Area (GB/SA) model ¹³⁴ as implicit solvation model method. The extended cut-off distances were set at their default values for implicit solvation, specifically Van der Waals (8.0 Å), electrostatic (20.0 Å) and H-bond interactions (4.0 Å). A 100 ps equilibration simulation was preceded by 2500 steps of minimization, followed by 2 ns production run and sample structure was recorded for every 10 ps of the simulation for trajectory analysis.

Explicit solvation simulation was performed using Desmond. The dendrimer glucosamine system was built using the SPC solvation model and the size of the box was determined automatically by creating a 10 Å buffer zone around dendrimer. The molecular dynamics simulation, structure minimization and relaxation steps were performed for 4.8 ns at 300K and 1.03 bar. Snapshot structures were recorded at every 4.8 ps.

The molecular surface was calculated for each structure in trajectories created by Maestro 9 using a 1.4 Å probe. The values were exported in the "cvs" file format. Flexibility was assessed with RMSD trajectory analysis in relation to the first structure using Maestro toolkit and exported in "cvs" format. The trajectories of both molecule were saved in "mol2" format and imported into VegaZZ 2.2.0.54 ¹⁷⁶ where the trajectory analysis tool was used for polar surface calculations along the trajectory with a probe radius of 1.4 Å. Microsoft Excel was used to plot all properties determined.

Results

Molecular dynamics simulations were performed with a generation 3.5 PAMAM dendrimer modified with eight glucosamine using implicit and explicit solvation. The resulting trajectories were used for comparison between the two methods. The properties determined were RMSD, total surface area and polar surface area as these were found relevant for the biological activity of the dendrimers.

Figure A6.2.1 displays the results found for RMSD calculation for both trajectories. It is possible to observe that trajectories are significantly different for the first 1.4 ns, this is because the initial structure was different for each simulation. Furthermore, the slope of RMSD changes for implicit solvation is steeper, which may be a result of explicit water molecule absence, since the movement of branches is not as restricted as in the case where the water molecules are explicitly present. However, after this initial divergence the trajectories for the dendrimer simulated with implicit and explicit solvation converged towards the end of the simulations.



Figure A6.2.1 RMSD calculations on two trajectories for a glycosylated gen.3.5 PAMAM dendrimer simulated using implicit (Blue) and explicit (Red) solvation methods.

The values for surface area and polar surface area for each trajectory were also determined (Figure A6.2.2 and A6.2.3). The results revealed no significant differences between the structures produced by the two different methods.



Figure A6.2.2 Surface Area values determined for two trajectories for a glycosylated gen.3.5 PAMAM dendrimer simulated using implicit (Blue) and explicit (Red) solvation methods.

A small variation is initially observed in the surface area values, this is most likely due to the fact that the simulation were started from different conformations (Figure A.6.2.2). However, from the same point where RMSD trajectories start to converge, at 1.4 ns the surface area values also become almost coincidental, showing there is no significant difference on the structures produced during the simulations using implicit and explicit solvation methods.



Figure A6.2.3 Polar Surface Area values determined for two trajectories for a glycosylated gen.3.5 PAMAM dendrimer simulated using implicit (Blue) and explicit (Red) solvation methods.

The polar surface area trajectories determined for the structures produced by dynamics simulations using different solvation methods are very similar throughout all the simulations time with approximately 7% difference between two trajectories (Figure A6.2.3).

Conclusion

The results above show there are no significant differences between the structures produced by the simulations using different solvation methods. Therefore, it was considered that the use of implicit solvation for the analysis of PAMAM, triazine and hybrid dendrimers could be performed efficiently with this method.