Designing dendrimers for use in biomedical applications

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CONTENTS

CHAPTER 1

DENDRIMERS FOR BIOMEDICAL APPLICATIONS

ABSTRACT

Dendrimers are highly branched, monodisperse molecules possessing a large number of end groups and can be synthesized in either a divergent or convergent manner. They adopt a globular shape and are polyvalent, two features that are often seen in naturally occurring systems. Therefore, dendrimers are regarded as promising candidates for use in biomedical applications. The toxicity of dendrimers is dependent on the functional groups at the periphery; while cationic dendrimers often show high toxicity, anionic or neutral dendrimers show little or no toxicity. Polyester-based dendrimers are biodegradable, whereas traditional dendrimers like poly(amidoamine) (PAMAM) and poly(propylene imine) (PPI) dendrimers show much lower biodegradability under physiological conditions. In recent years, dendritic scaffolds have been explored for their use in biomedical applications such as drug delivery, synthetic vaccination, magnetic resonance imaging or tissue engineering. These initial results are promising and serve as a foundation for the research described in this thesis.

1.1. DENDRIMERS

1.1.1. STRUCTURAL CHARACTERISTICS

Dendrimers, also known as cascade molecules, arborols, cauliflower, or starburst polymers are attractive building blocks because of their unique structures and properties.[1-6] The term dendrimer (Greek: *dendron* = tree, *meros* = part) graphically describes the architecture of this class of molecules. Dendrimers exploit two traits that are frequently observed in many naturally occurring systems: globular structure and polyvalency. In addition, they are monodisperse macromolecules with a regular and highly branched three-dimensional architecture.[6-8] The first dendritic wedge was reported by Denkewalter *et al.*[9] using lysine residues as branching units (section 1.2.2). Since the pioneering work of Vögtle,[10] Tomalia[11] and Newkome[12] on dendrimer synthesis in the early 1980s, several research groups have contributed to both the development of synthetic methodology as well as specific applications of dendrimers.[3,13] One of the most appealing features of dendrimers is the existence of a high number of functional groups at the periphery, by which the properties of these macromolecules can be modulated.[1,3,6,14,15] Therefore, dendrimers can easily be used as polymeric materials in fields such as catalysis,[16] chiral recognition,[17] unimolecular micelles,[18] host–guest chemistry,[1,3,19,20] light-harvesting,[21-23] and liquid crystalline materials.[24-31] The structural diversity in the repeat units is impressive, ranging from pure hydrocarbons to peptides to coordination compounds. The two most commonly used dendrimers, the poly(amido amine) dendrimers (PAMAM) and the poly(propylene imine) dendrimers (PPI), have been produced industrially and are commercially available. In this research, divergently produced poly(propylene imine) dendrimers are used which have been reported independently by Mülhaupt[32] and De Brabander.[33]

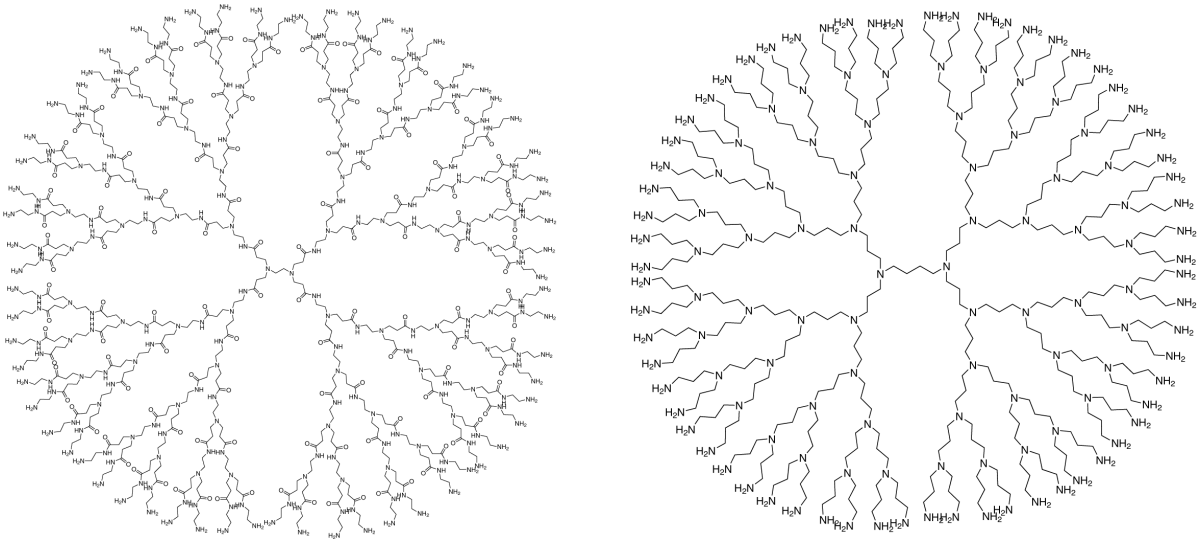

Figure 1.1 *Structural formula of poly(amido amine) dendrimer (PAMAM) and poly(propylene imine) dendrimer (PPI), both are commercially available.*

1.1.2. SYNTHESIS

Dendrimers are multivalent macromolecules with a regular, highly branched structure and dimensions resembling those of small proteins. These structures are synthesized via a cascade synthesis using an iterative sequence of reaction steps. Two main synthetic strategies for synthesizing dendrimers have emerged: the divergent and convergent approach.

In the divergent approach, pioneered by Newkome and Tomalia, the growth of a dendrimer originates from a core site, or root. Each repetition cycle leads to the addition of one more layer of branches — called a generation — to the dendrimer framework. Therefore, the generation number of the dendrimer is equal to the number of repetition cycles performed, and may be easily determined by counting the number of branch points as one proceeds from the core to the periphery.[10,12,32-35]

The poly(propylene imine) dendrimers (PPI) used in this research have been developed at DSM and are built from a 1,4-diaminobutane core with tertiary amines serving as the branch points (Figure 1.2).[33] The reaction sequence for the synthesis of poly(propylene imine) dendrimers is a repetition of a Michael addition between acrylonitrile and primary amines, followed by the heterogeneously catalyzed hydrogenation of the nitriles, resulting in a doubling of the number of primary amines. Both reaction steps in the sequence can be carried out to extremely high conversions with high selectivity.

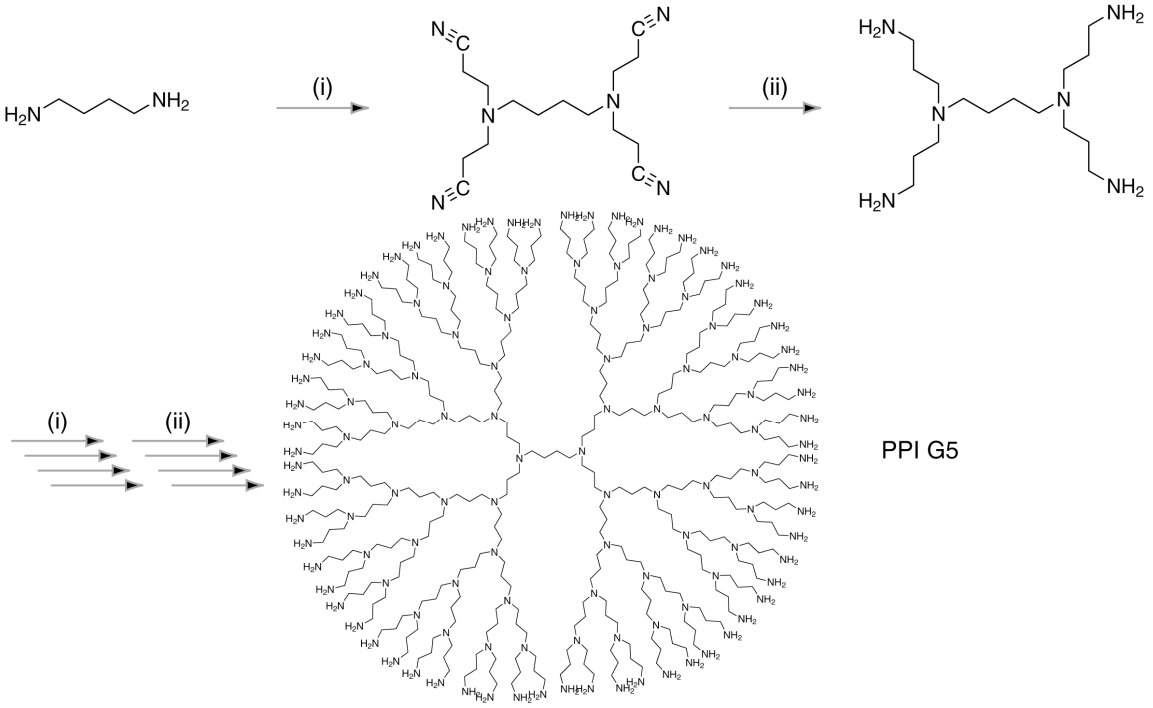

Figure 1.2 *Divergent synthesis of poly(propylene imine) dendrimers. Reaction conditions: (i) acrylonitrile; (ii) hydrogen gas, Raney Cobalt. After the first two steps, the first generation dendrimer is obtained, repeating these steps results in higher generation dendrimers (5th generation is shown: PPI G5).*

Conversely, in the convergent approach as pioneered by Hawker and Fréchet, synthesis proceeds from what will become the dendron molecular surface (periphery) inward to a central core (focal point).[36-41] As the intermediate products in the convergent approach are easier to purify, dendrimers prepared by this method are generally considered to be more homogeneous (often monodisperse) than those prepared by the divergent approach. On the other hand, the convergent strategy is often limited to dendrimers of lower generation numbers.

1.2. BIOLOGICALLY ACTIVE DENDRITIC STRUCTURES

The following sections describe the approach to make dendritic macromolecules (with a focus on glycodendrimers and peptide dendrimers) that are potentially biologically active. These dendrimers consist of two parts: (1) a core or a template bearing several branching segments that give the molecule a unique spatial architecture and dendritic character, and (2) multiple copies of biologically active structures (*e.g.* peptide antigens and saccharides attached onto the core, that provide the molecule with designed biological properties, respectively). One approach to synthesize these dendrimers is to functionalize the dendritic templates (PAMAM or PPI) at the periphery with biologically active molecules. Another method is to construct dendrimers that have biologically active molecules as building blocks.

1.2.1. GLYCODENDRIMERS

Glycodendrimers are dendrimers that incorporate carbohydrates in their structure and can be classified into three types: as carbohydrate-coated dendrimers, carbohydrate-centered dendrimers or carbohydrate-based dendrimers.[42-44] The investigations in carbohydrate-coated dendrimers have largely focused on two classes of dendrimers, the PAMAM dendrimers[11] and PPI dendrimers.[33] Both dendrimers have been modified with glycosyl derivatives by means of urea, thiourea and amide bonds.[45,46] Cyclodextrins could provide interesting cores for carbohydrate-centered dendrimers as they would allow combination of multivalent structures with the ability to complex small hydrophobic molecules within the cavity of the cyclodextrin moiety (Figure 1.3).[47] Molecules of this type could therefore be designed for use in targeted drug delivery. There are numerous examples of carbohydrate-based dendrimers synthesized via native glycosidic linkages,[48,49] peptide coupling reactions,[50] or reductive amination.[51,52]

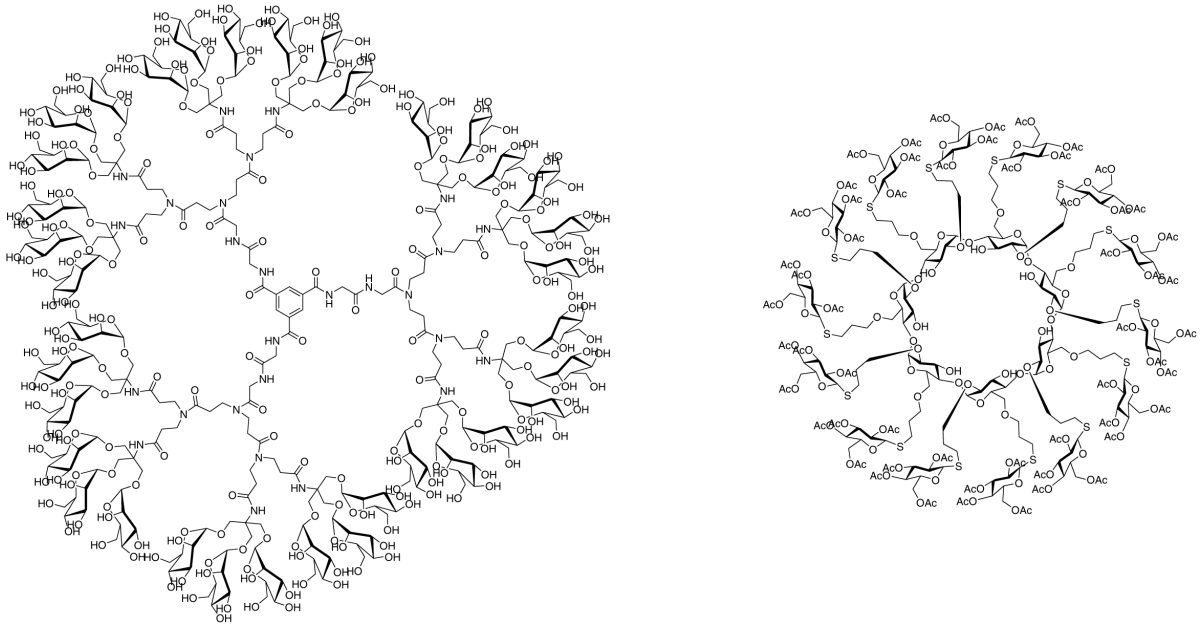

Figure 1.3 *Structural formula of a carbohydrate-coated dendrimer bearing 36 α-D-mannopyranosyl residues (left)*[51,52] *and a carbohydrate-centered dendrimer having a β-cyclodextrin core and 14 copies of a glycosylthiol residue (right).*[47]

Glycodendrimers have been used for a variety of biologically relevant applications. Most notably, glycodendrimers with surface carbohydrate units have been used to study the protein-carbohydrate interactions that are implicated in many intracellular recognition events. Here, multivalent interactions are important to obtain relevant affinities and, therefore, dendrimers are attractive for the study of protein-carbohydrate interactions. Compared with other frameworks that have been used to study such interactions, dendrimers are appealing because of their size and low polydispersity. Furthermore, the sizes of glycodendrimers can be varied in a controlled manner depending on the generation of dendrimer used. In a recent review, the carbohydrate binding properties of many such glycodendrimers are discussed.[42]

1.2.2. PEPTIDE DENDRIMERS

Peptide dendrimers can be generally defined as highly branched structures of non-natural origin that contain peptide bonds. Similar to glycodendrimers, peptide dendrimers can be divided into three types. Trifunctional amino acids can be used as branching points resulting in the first type of peptide dendrimers (type I). Moreover, these dendrimers can be functionalized at the periphery with peptide chains (type II). Grafted peptide dendrimers (type III) are conventional dendrimers with either unnatural amino acids or organic groups as the branching core and peptides or proteins attached as surface functional groups.

By virtue of their dendritic architecture, peptide dendrimers hold promise in various biochemical and biomedical applications, *e.g.* as synthetic vaccines, adjuvants, artificial protein-like structures, in mimicking ion-channel structure, in the study of inter-cellular

interactions and as drug carriers. Peptide dendrimers vary from low molecular weight species of 2 kDa to large protein–like constructs of more than 100 kDa. Similar to other dendrimers, synthesis of peptide dendrimers is controlled with products of consistent size, architecture and composition. Some examples of the three types of peptide dendrimers and their possible applications will be discussed. The names, structural formulas, three-letter code and one-letter code abbreviation of all naturally occurring amino acids can be found in the appendices. For convenience, the one-letter code abbreviation of amino acids will be used throughout this thesis.

1.2.2.1 POLY(AMINO ACID)S AS BRANCHING UNITS

Amino acids are frequently used as building blocks for the synthesis of peptide dendrimers. By virtue of their trivalency, reactivity and synthetic expedience, branching units derived from lysine and its homologs are the most popular (Figure 1.4). In the early 1980s, Denkewalter patented the synthesis of L-lysine-based dendrimers up to the tenth generation.[9,35,53] The diamino nature of lysine creates a situation where each additional level of lysine effectively doubles the number of sites to which peptide monomers may be attached. A typical branching lysine unit consists of three lysines to give a tetravalent peptide dendrimer (Lys_2Lys = K_2K, Figure 1.4). Further divergence of the K_2K unit to one or two additional levels will generate di-K_2K and tetra-K_2K dendrons with reactive ends of 8 and 16 amino groups, respectively, to which peptides can be attached. Peptides may be synthesized directly onto the lysine dendron using a divergent strategy or dendrons can be further functionalized with electrophiles and thiol nucleophiles for convergent ligation of peptides. Many variations of K_2K cores have evolved.[54-64]

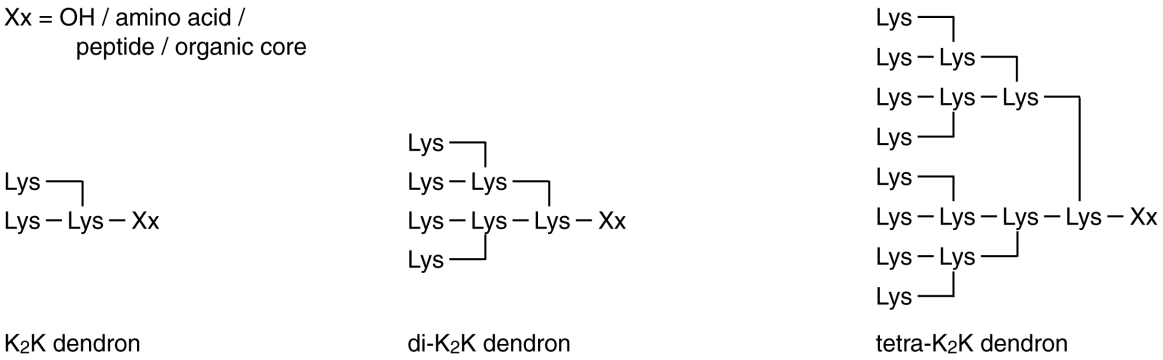

Figure 1.4 *Schematic representation of polylysine cores consisting of K_2K, di-K_2K and tetra-K_2K. An increase in the number of lysine branching units increases the number of surface amine groups.*

Other examples of peptide dendrimers with amino acids as branching units, include the peptide dendrimers containing L-leucine or L-valine as monomers as developed by Zimmerman *et al.*[65] Furthermore, Hirsch *et al.* developed new chiral dendrimers known as depsidendrimers, owing their structural resemblance to depsipeptides (Figure 1.5).[66,67]

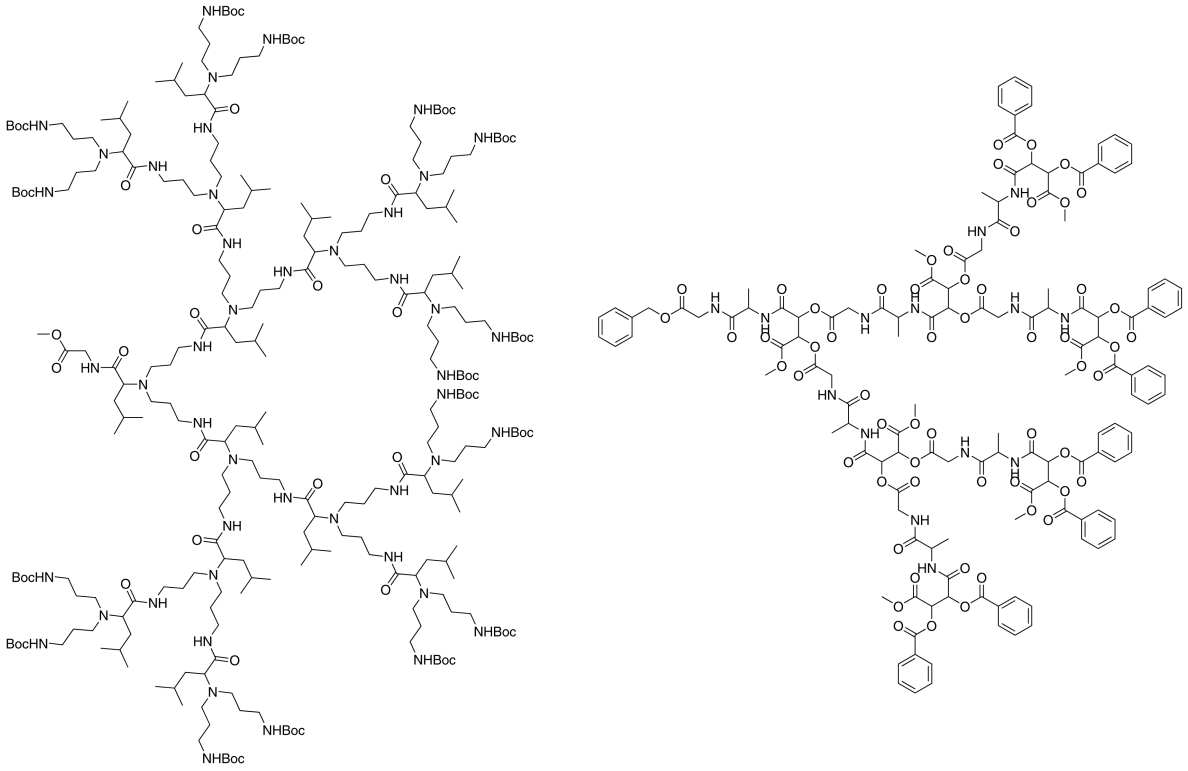

Figure 1.5 *Structure of L-leucine dendrimer developed by Zimmerman* et al.[65] *(left). Instead of L-leucine also L-valine can be used. Structure of a third generation depsipeptide dendrimer developed by Hirsch* et al. *(right).*[66]

With the aim of designing simple models for globular protein mimics, a collection of polyglutamic dendrimers of various generations has been synthesized.[68-70] A 1,3,5-benzenetricarbonyl unit was used as a central core in order to induce cylindrical spatial arrangement of the dendrons by virtue of its threefold symmetry. This unit may also promote intermolecular association through either π–π stacking or amide–amide hydrogen bonding. Dendritic polyglutamic porphyrins have been prepared in order to probe the porphyrin-based phosphorescence for *in vivo* oxygen measurements.[71] With the aim of obtaining new chiral HPLC stationary phases, enantiomerically pure L-glutamic acid monomer-based dendrimers have been developed and their subsequent immobilization onto chromatographic supports has been studied.[72,73] Peptide dendrimers based on polyproline helices have been synthesized in order to explore the helical conformational of the polyproline chains,[74] as polyproline oligomers are known to adopt a right-handed helix in organic solvents[75] and a left-handed helix in aqueous solvents.[76] Another example of proline-containing dendrimers has been described by Goodman *et al.*, in which collagen-mimetic dendrimers exhibit enhanced stability compared to that of equivalent scaffold-terminated structures (Figure 1.6).[77]

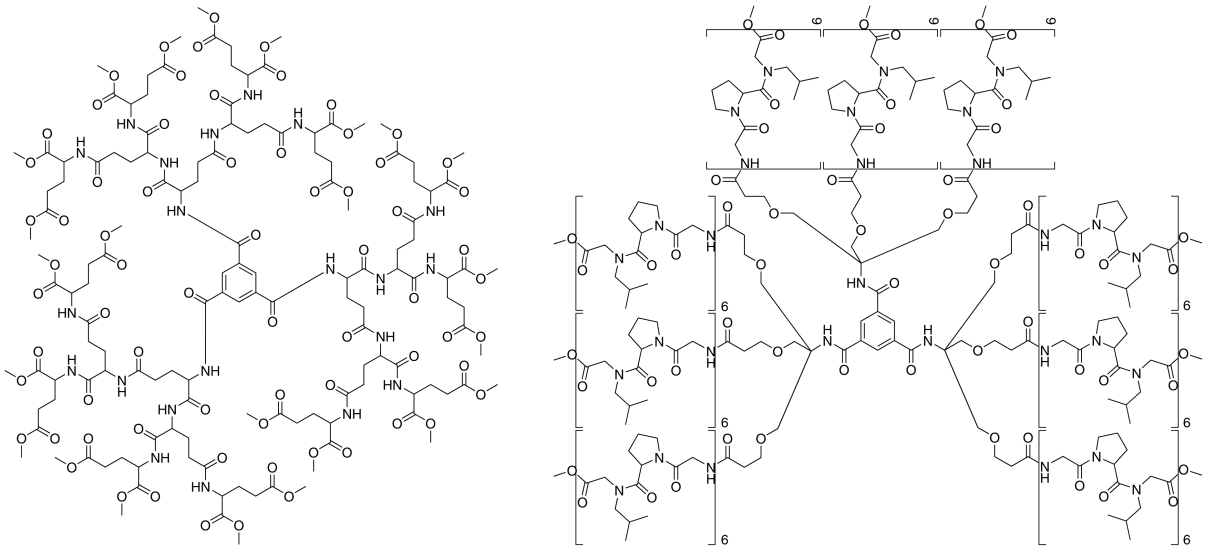

Figure 1.6 *Left: Structure of polyglutamic dendrimer using a benzene core.*[68-70] *Right: Structure of a collagen-mimic dendrimer as designed by Goodman* et al.[77]

1.2.2.2 AMINO ACID BRANCHING UNITS AND SURFACE PEPTIDYL CHAINS

Inspired by the work of Denkewalter, Tam pioneered the synthesis and study of multiple antigenic peptides (MAPs), a distinct type of dendrimer.[57,78-82] MAPs with two, four, eight, or sixteen copies of synthetic peptide antigen can be produced by utilizing oligolysine cores with one to four sequential levels of lysine residues. Although the higher-level analogs are difficult to prepare, they are very useful because they significantly improve immunogenicity.[83] A broad range of peptide dendrimers based on MAP scaffolds with a varying degree of branching has been synthesized using solid phase peptide synthesis (SPPS).[79-81] The use of chemoselective ligation approaches to introduce the antigenic peptide segments[78,84,85] or the glycopeptide antigens[86-89] has improved the preparation of MAPs. Apart from their use in vaccine design, the multimeric construction of MAPs has garnered interest in other areas,[90-99] such as immunoassay and serodiagnosis reagents,[100] inhibitors,[101] epitope mapping reagents,[102] artificial proteins,[103] protein mimics,[104] intracellular delivery agents,[105-110] supramolecular gels,[111-113] and purification reagents.[114]

1.2.2.3 GRAFTED PEPTIDE DENDRIMERS

Only a few examples exist of peripheral attachment of peptides to dendritic scaffolds, like the PAMAM dendrimers. Bradley *et al.* developed an efficient solid-phase synthesis of PAMAM dendrimers to produce homogeneous molecules.[115] Furthermore, these products were shown to be suitable starting points for the solid-phase synthesis of peptide-modified dendrimers up to six amino acid residues in length. In addition, peptide dendrimers have been designed and synthesized as artificial photosynthetic systems, whereby amphiphilic right-handed helix peptides were introduced at the end groups of PAMAM dendrimers through a thioether linkage.[116-118] A direct method for the formation of peptide and carbohydrate dendrimers has been successfully developed in which PAMAM dendrimers are covalently

modified via a convergent approach with either peptide or carbohydrate molecules.[119,120] Other examples of periphery modification of dendrimers with peptides have been shown by Higashi *et al.*,[121,122] in which helical polyglutamic peptides where assembled on a PAMAM dendrimer surface by graft polymerization. The resultant peptide dendrimers were shown to have enhanced peptide-segment helicity as compared to that for nondendrimer-based analogs, a fact attributed to aggregation of the peptide segments on the dendrimer surface.[123]
The few reports of protein ligation to dendrimers typically involve the coupling of a single protein using nonspecific conjugation chemistry that results in conjugation at multiple sites on the protein. Reacting a PAMAM core with calf intestine alkaline phosphatase and a Fab fragment of an anti-creatine kinase MB isoenzyme antibody generated a biochemical reagent.[124] Recently, the preparation of multivalent hemoglobin dendrimers was reported that involved specific conjugation of a fourth generation dendrimer to hemoglobin using a hemoglobin-specific cross-linking agent.[125]

1.3. BIOCOMPATIBILITY OF DENDRIMERS

Dendrimers have to fulfill certain requirements to be able to apply them in drug design or as a drug delivery vehicle *in vivo*. For instance, they should be non-toxic, able to cross biological barriers, able to circulate in the body for the time needed to have a clinical effect and they should be able to target to specific structures. Moreover, these characteristics must be accompanied by a reasonably fast renal elimination rate or biodegradation rate.
The nature of a dendrimer's numerous end groups dictate whether or not it displays a significant toxicity. A difference was observed between the toxicity of cationic dendrimers and neutral or anionic dendrimers. PAMAM and PPI dendrimers with terminal primary amines generally display concentration-dependent toxicity and hemolysis,[126-128] whereas dendrimers containing neutral or anionic groups have been shown to be much less toxic and hemolytic.[127,129-132] Partial or complete modification of the periphery of the cationic dendrimer with negatively charged or neutral groups results in a lower toxicity.[127,128,133] The toxicity of cationic PAMAM dendrimers increases with each generation, but surprisingly, cationic PPI dendrimers do not follow this trend.[126,127] Although both PAMAM and PPI dendrimers have primary amino groups at the periphery, their interior structure is markedly different (Figure 1.1) which may contribute to these toxicity effects. In addition, the molecular weight of each dendrimer type for a given number of end groups is so different that this may also be a contributory factor. The cytotoxicity of the cationic dendrimers can be explained by the favored interactions between negatively charged cell membranes and the positively charged dendrimers surface, enabling these dendrimers to adhere to and damage the cell membrane, causing cell lysis.
In vivo toxicity correlated reasonably well with *in vitro* toxicity. Only a few systematic studies on the *in vivo* toxicity of dendrimers have been carried out so far. In general mice tolerate low

doses of positively charged PAMAM dendrimers, independent of whether they are modified at the dendrimer surface or not.[126,134] Neutral polyester dendrimers have shown to be non-toxic both *in vitro* and *in vivo*.[130,135] As the dendrimer may be degraded by hydrolytic enzymes after the release of the drug, this makes them very promising as biodegradable drug delivery devices.

The biopermeability of dendrimers is of importance when dendrimers are considered as potential drug delivery vehicles. It has been shown by *in vivo* studies that the extravasion time across the microvascular endothelium increases with increasing generation and molecular weight of the cationic amino-terminated PAMAM dendrimer.[136] The transfer rate of anionic PAMAM dendrimers was faster than other polymeric systems.[137] Biodegradability of dendrimers can prevent bioaccumulation and accompanying toxic effects. Hydrolysis of PAMAM dendrimers is slow under physiological conditions, and polyester dendrimers are more promising in this regard. Furthermore, dendrimers with thiol-reactive disulfides have been prepared that can be reduced and degraded inside cells. There are a few ingenious examples of degradable dendrimers reported recently, including self-immolative,[138] cascade-release[139] (Figure 1.7) or geometrically disassembling[140,141] dendrimers. In these dendrimers, a single reaction initiates the complete disassembly into small structurally similar units. Importantly, this disassembly strategy not only results in complete and rapid dendrimer degradation, but also provides a means for release of multiple biologically active species.

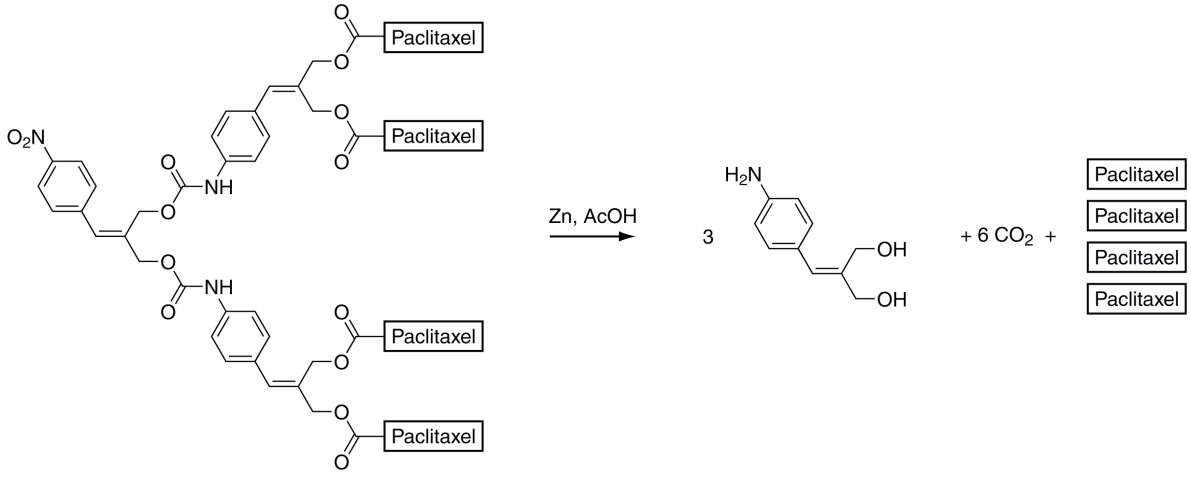

Figure 1.7 *"Cascade-release dendrimers".[139] Release of four paclitaxel leaving groups upon reduction.*

1.4. BIOLOGICAL APPLICATIONS OF DENDRIMERS

1.4.1. DRUG AND GENE DELIVERY

A few *in vivo* studies on dendrimer-based drug carriers have been reported. Cisplatin was complexed to the surface groups of a carboxylate-terminated PAMAM dendrimer which led to a tenfold increase in the solubility of cisplatin compared to the free drug.[142] The conjugates were shown to target to subcutaneous tumors in mice via the enhanced permeation and retention effect.[143,144] A fatty acid functionalized PAMAM dendrimer was shown to bind 5-

fluoracil, a water-soluble anti-tumor drug, and the oral bioavailability in rats was nearly twice the level of free 5-fluoracil.[145] PAMAM dendrimers have also been used as antitumor targeted carriers of methotrexate.[146] The dendrimer was modified with folate as targeting ligand, a fluorophore and methotrexate and injected in mice. The concentration of dendrimer in the tumor was five to ten times higher than the control without the folate ligand. Furthermore, it significantly reduced the rate of tumor growth. Several other dendritic drug devices have been developed, *e.g.* dendrimers as antiviral drugs[147-149] and antibacterial drugs.[150] Gene delivery has been accomplished using a variety of positively charged dendrimers to form DNA complexes and transfect cultured cells with lower toxicities and higher efficiencies than conventional transfection agents.[151-157]

1.4.2. VACCINES

Examples of peptide dendrimers being used for vaccine and immunization purposes include the multiple antigenic peptide (MAP) dendrimer system pioneered by Tam *et al.*[56,78,79] which can be synthesized with defined mixtures of B- and T-cell epitopes. An example of this is described by Moreno *et al.* in which a MAP construct was modified with *Plasmodium falciparum* T- and B-cell stimulatory peptides.[158,159] Using cancer related peptides Ota *et al.* showed that MAPs were processed in antigen presenting cells in the same way as antigens derived from intracellular pathogens (*e.g.* viruses) thereby providing a powerful immune response, including cytotoxic T-cells.[160]

1.4.3. TISSUE ENGINEERING

Grinstaff *et al.* have shown that dendrimers' high functional-group densities and low solution-viscosities make them useful as injectable sealants for corneal wounds.[161-163] In this work, the peripheries of biodegradable polyester dendrimers are functionalized with reactive groups capable of crosslinking and forming and an insoluble hydrogel matrix upon activation, *e.g.* polymerization with ultraviolet light. Using this procedure, the authors have successfully sealed experimental corneal lacerations and are exploring the use of these materials for additional ophthalmic surgeries.

1.4.4. IMAGING

The earliest *in vivo* uses of dendrimers were as carriers for magnetic resonance imaging (MRI) contrast agents[164,165] and several reviews exist on the application of dendrimers in diagnostics.[166,167] PAMAM dendrimers have been modified with the chelating ligand Gd(III)-DTPA (DTPA = diethylenetriaminepentaacetic acid) through a thiourea linkage. *In vivo* experiments showed excellent MRI images of blood vessels and long blood circulation times (>100 min) upon intravenous injection of these dendrimer conjugates with gadolinium. Also PPI dendrimers have been modified with Gd(III)-DTPA ligands.[168] Related to this research, gadomer 17 has been introduced, a dendrimer based on a 1,3,5-benzoic acid core and lysine residues as branching units with Gd-DTPA moieties attached. Gadomer 17 shows a good elimination rate, presumably as a result of the globular nature of the dendrimer derivative. In

addition, dendrimers for use in MRI have been developed that include both targeting and imaging components.[169]

1.5. AIM AND OUTLINE OF THIS THESIS

Literature shows that due to their multivalency and globular shape dendrimers are regarded as ideal candidates for use in biomedical applications. A number of challenging targets remain in the design and synthesis of dendrimers for this purpose. In this thesis, the supramolecular encapsulation of guest molecules in the interior of a dendrimer is evaluated by using specific interactions (electrostatic, hydrophobic and hydrogen bonding). Furthermore, a general strategy for the efficient attachment of peptides to the periphery of dendrimers is evaluated. Once these dendrimers are prepared the bioactivity *in vitro* and *in vivo* is explored.

In Chapter 2, the synthesis of a new class of dendrimers is described that can be applied in drug delivery. Poly(propylene imine) dendrimers are modified with ethylene glycol chains for non-toxicity and enhanced water-solubility and the dendrimers are subsequently tested for their toxicity *in vitro*. The dendrimers possess a hydrophobic domain that is able to encapsulate small apolar molecules. Investigation was done on the encapsulation of the dye Rose Bengal, a complex based on electrostatic interactions. In addition, the encapsulation of an anti-inflammatory drug cortisol was investigated; this complex formation was based on hydrophobic interactions. To strengthen complex formation, a cortisol prodrug was synthesized that has several secondary interactions with the dendrimer, such as electrostatic, hydrophobic, and hydrogen-bonding interactions. The release of the drug from the carrier was studied and the bioactivity of the drug was measured *in vitro*.

Chapter 3 describes the synthesis and characterization of multivalent peptide dendrimers. Peptide dendrimers are synthesized via native chemical ligation of a *C*-terminal thioester-peptide with an *N*-terminal cysteine-dendrimer. First, second and third generation are prepared and the ligation of a range of peptides has been investigated in detail. Efficient modification of the resulting peptide dendrimers for use in biomedical applications is still an unresolved issue in literature. Different chemical strategies for the introduction of functionalities, such as the drug cortisol, MRI probes and fluorescent probes are evaluated in Chapter 4. The first approach uses disulfide bridges and in the second approach thiol groups are reacted with maleimide probes. The last approach that is described focuses on oxime chemistry.

After the synthesis and characterization of peptide dendrimers has been studied in detail, the biological application of the resulting peptide dendrimers is evaluated in Chapter 5 in a first and preliminary study. The *in vivo* studies include the application of peptide dendrimers as a synthetic vaccine using two approaches. Rabbits are vaccinated with dendrimers that are modified with part of the parasite cirumsporozoite protein to test the potential of the

dendrimers as a synthetic vaccine against malaria. In addition, rats are vaccinated with dendrimers that are modified with the gonadotropin releasing hormone to test the potential as a synthetic vaccine against prostate cancer. Preliminary studies of peptide dendrimers *in vitro* include the multivalent binding of a peptide dendrimer to a receptor-coated surface. For this purpose, the arginine-glycine-aspartic acid (RGD) amino acid sequence is attached to a dendrimer and binding studies are performed on an integrin $\alpha_V\beta_3$-coated surface. The β-amyloid peptide plays an important role in Alzheimer's disease. Therefore, the periphery of a dendrimer is modified with the peptide sequence lysine-leucine-valine-phenylalanine-phenylalanine (KLVFF) and the influence of the resulting peptide dendrimer on aggregation and toxicity of the β-amyloid peptide is investigated.

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CHAPTER 2

DESIGNING DENDRIMERS FOR DRUG DELIVERY

ABSTRACT

A convenient methodology has been developed for the synthesis of water-soluble dendrimers possessing a hydrophobic domain next to the basic core. The dendrimers are not toxic to rat macrophages. They are able to form complexes with the dye Rose Bengal through electrostatic interactions and with the drug cortisol through hydrophobic interactions. To strengthen the complex formation of dendrimer and drug, cortisol was modified with a urea-glycine tail in order to have a combination of multiple interactions (electrostatic, hydrophobic and hydrogen bonding) between dendrimer and cortisol. From this cortisol prodrug, the active cortisol is expected to be released by either enzymatic or basic cleavage of the ester bond. Dissolving the prodrug in water in the presence of the dendrimer resulted in an almost six-fold increase in solubility. Both cortisol and the cortisol prodrug, as well as dendrimer-drug complexes, show similar biological activities, indicating the release of active cortisol from the carrier and degradation of the ester bond in case of the prodrug.

2.1. DENDRIMERS FOR DRUG DELIVERY

Dendrimers can be used as potential carriers for (targeted) drug delivery in at least two ways. Biologically active molecules can be either physically entrapped inside the dendritic structure (described in this chapter), or covalently attached to the periphery of the dendrimer to yield dendrimer-drug conjugates (Chapters 3 and 4). The dendritic cavity of an appropriately designed dendritic structure could be used for trapping drugs with the possibility of subsequent controlled release. Studies by several research groups have shown that the interior of a dendrimer is capable of encapsulating guest molecules.[1-18] These examples illustrate that the concept of using host-guest chemistry for drug delivery may be feasible.

Here, the design, synthesis and characterization of a new class of poly(propylene imine) dendrimers are described ((Figure 2.1, section 2.2). The dendrimers were modified at the periphery with ethylene glycol groups, which are commonly used groups in biomedical applications because of their good water solubility, low toxicity and high biocompatibility.[19] One of the dendrimers has a hydrophobic domain for enhancing hydrophobic interactions between guest (encapsulated small molecules) and host (dendrimer). In addition, the dendrimers were tested for their application as tools for drug delivery, by assessing the sub-acute (48 hours) *in vitro* cellular toxicity of the dendimers towards rat macrophages. Host-guest properties of the dendrimers were investigated by encapsulation of the dye Rose Bengal.

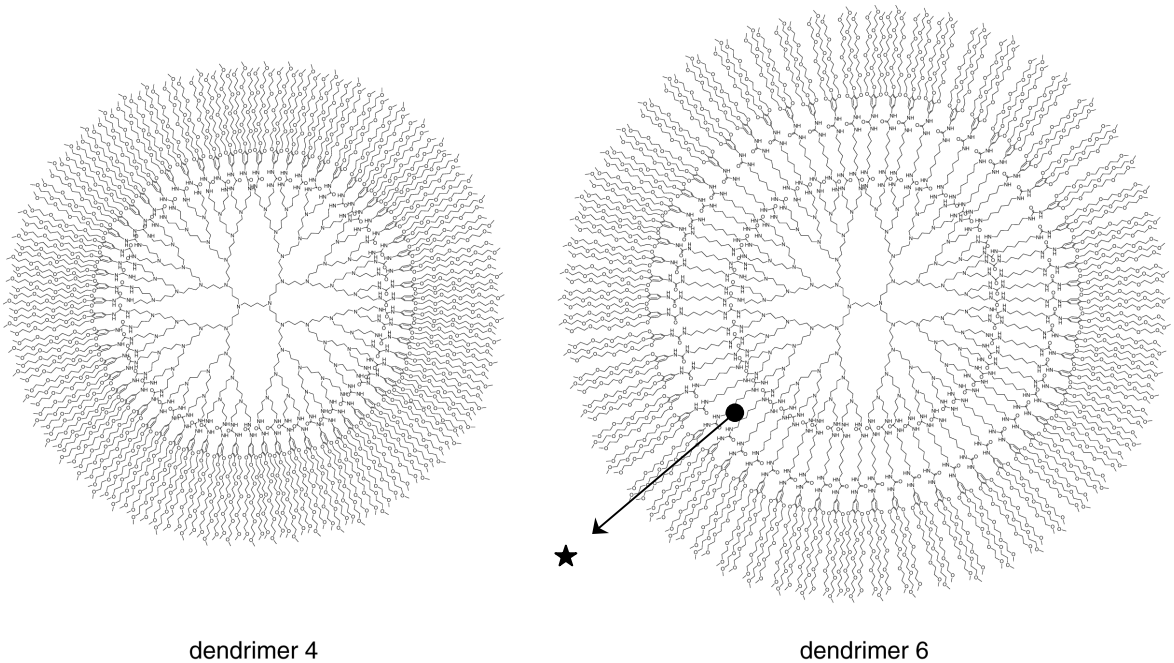


Figure 2.1 *Schematic representation of novel dendrimers 4 and 6, showing the hydrophobic domain in dendrimer 6 (indicated with a star).*

As a model system to evaluate our novel dendrimers, the encapsulation of the low molecular weight drug cortisol was investigated (section 2.3). Cortisol is a natural glucocorticoid hormone, synthesized in the adrenal cortex. It is widely used as a drug because of its anti-inflammatory action. Like other (synthetic) glucocorticoids, its long term use is associated with unwanted side effects such as increased risk for osteoporosis and adrenal insufficiency.[20] Applying the drug locally at the site of desired action (*e.g.* the site of inflammation) would greatly reduce the required dose, and thereby the risk of side effects.[20] Here, it was evaluated whether water-soluble dendrimers are suitable carriers for local drug application using the hydrophobic drug cortisol as a model compound and by evaluating the pharmacological action of the dendrimers loaded with this glucocorticoid drug.

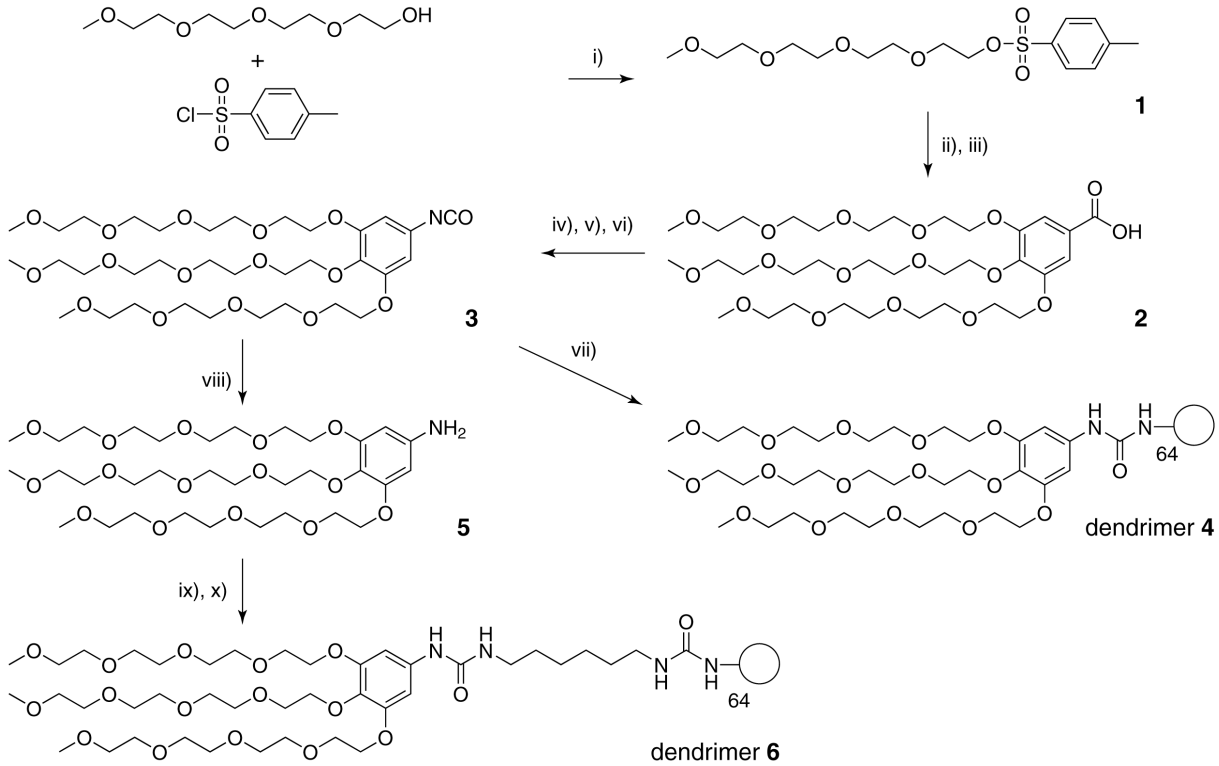
2.2. NOVEL NON-TOXIC WATER-SOLUBLE DENDRIMERS

The synthesis of a new class of poly(propylene imine) dendrimers is developed. The dendrimers are modified at the periphery with ethylene glycol chains and one of the dendrimers has a hydrophobic domain for encapsulation of small apolar compounds such as drugs. The dendrimers are tested for their toxicity using rat macrophages. Furthermore, host-guest properties are investigated by encapsulation of the xanthene dye Rose Bengal.

2.2.1 SYNTHESIS

The poly(propylene imine) dendrimers used in this research have been developed at DSM and are built from a 1,4-diaminobutane core with tertiary amines as branching units (Chapter 1).[21] Non-modified poly(propylene imine) dendrimers are known to be toxic due to the free amine groups at the periphery. However, modification of the dendrimer at the periphery considerably reduces toxicity to negligible or irrelevant levels.[22]

For the synthesis of dendrimers **4** and **6**, a solution of *p*-tosylchloride in THF was added dropwise at 5 ^{o}C to a solution of tetra(ethylene glycol) monomethyl ester and sodium hydroxide in water, yielding the activated tetra(ethylene glycol) **1** in 93% yield (Scheme 2.1). Compound **1** was added dropwise to methyl gallate and refluxed overnight in order to couple the ethylene glycol groups to the gallic acid scaffold. Subsequent hydrolysis of the methyl ester rendered the tetra(ethylene glycol) functionalized gallic acid **2** in 83% yield. Reaction of **2** with ethyl chloroformate, subsequent addition of sodium azide and performing a Curtius rearrangement in refluxing dioxane, yielded the isocyanate **3** (reactions were monitored by IR spectroscopy), which was directly used in further synthesis for stability reasons. Addition of isocyanate **3** to the fifth generation of poly(propylene imine) dendrimer (64 end groups) was performed in chloroform and monitored by IR spectroscopy. Structural characteristics of the ethylene glycol functionalized dendrimer **4** with ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, IR spectroscopy, and elemental analysis revealed that the pure compound was obtained in 81% yield.



Scheme 2.1 *Synthesis of the fifth generation of dendrimer* **4** *and dendrimer* **6**. *Only the end group is shown, the circle represents the non-modified dendrimer. Reaction conditions: i) NaOH, THF; ii) methyl gallate,* K_2CO_3*, MIBK; iii) NaOH, MeOH,* H_2O*; iv) ethyl chloroformate,* Et_3N*, THF; v)* NaN_3*,* H_2O*; vi) heat, dioxane; vii) G5 PPI dendrimer,* $CHCl_3$*; viii)* Bu_4NOH*,* H_2O*, dioxane; ix) hexamethylenediisocyanate, toluene; x) G5 PPI dendrimer,* $CHCl_3$.

For the synthesis of dendrimer **6**, hydrolysis of isocyanate **3** was carried out by dropwise addition of a solution of **3** in dioxane at 90 °C to a solution of tetrabutylammonium hydroxide in water, yielding the corresponding amine **5**. A solution of amine **5** in toluene was added dropwise to a large excess of hexamethylene diisocyanate in order to obtain an apolar spacer (hydrophobic domain) between the gallic acid group and the dendrimer. After precipitation in hexane to remove excess of hexamethylene diisocyanate, a solution of the fifth generation of poly(propylene imine) dendrimer in toluene was added and dendrimer **6** was obtained in good yield (85%) and characterized with ^{1}H NMR, ^{13}C NMR, IR spectroscopy, and elemental analysis.

Two classes of water-soluble dendrimers have been synthesized in good yields. One of the dendrimers has a hydrophobic domain for encapsulation of small apolar molecules. The dendrimers are designed for use in drug delivery and are therefore tested for their toxicity on rat macrophages.

2.2.2 TOXICITY TESTS

To measure the toxicity of dendrimers **4** and **6**, rat macrophages (NR8383) were incubated with stepwise increasing concentrations of dendrimer for 48 hours. In case of dendrimer **4** concentrations of 0, 0.3, 1, 3, 10, and 30 μM were measured, while dendrimer **6** was measured

in concentrations of 0, 0.01, 0.1, 1, 10, and 100 μM. After the incubation, three tests were carried out; the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-terazoliumbromide (MTT) test, which probes the viability of the mitochondria; a trypan blue exclusion (TBE) test in which only dead cells are stained as intact membranes are not permeable to the dye trypan blue; and an NADH permeation test, in which the viability of the cells is measured by the amount of enzymes that have leaked out through the cell membrane (experimental section). The results are plotted relative to the values obtained when no dendrimer was present in the medium (Figure 2.2).

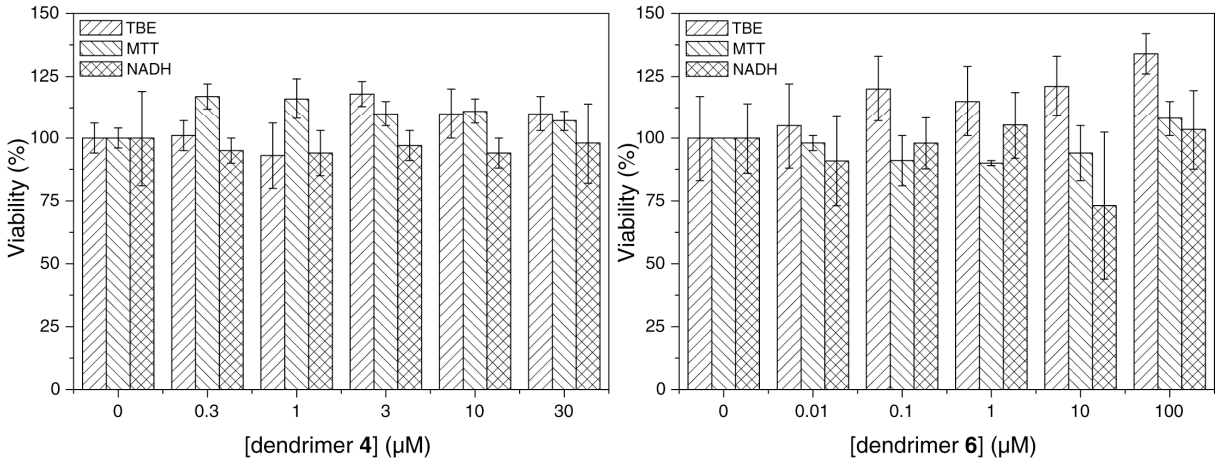


Figure 2.2 *Viability of rat macrophages after incubation with dendrimer* ***4*** *(left) and dendrimer* ***6*** *(right); results of MTT-test (MTT), trypan blue exclusion test (TBE) and NADH permeation test (NADH). Error bars are standard deviations.*

In the tests with different concentrations of dendrimer **4**, the viability of the macrophages reached values between 95-110%, showing a high viability (Figure 2.2, left). For dendrimer **6** the MTT test and the trypan blue exclusion test show viabilities of macrophages between 95-100% (Figure 2.2, right). The NADH permeation test in presence of dendrimer **6** showed viabilities of macrophages between 75-100%. These results clearly indicate that both dendrimers were not toxic to rat macrophages in concentrations up to 30 μM (dendrimer **4**) and 100 μM (dendrimer **6**). Concentrations higher than 30 μM (dendrimer **4**) and 100 μM (dendrimer **6**) were not measured. The toxicity of higher concentrations of dendrimer **4** and **6** was therefore unknown.

Some (proinflammatory) compounds are able to induce macrophage proliferation, which is undesirable and should be checked when using these compounds in biomedical applications. Both dendrimer **4** and **6** showed similar results in the toxicity tests (Figure 2.2) and are expected to yield similar results in macrophage proliferation. For that reason, only dendrimer **6** was studied for inducing macrophage proliferation. Macrophages were incubated with increasing concentrations (0, 0.01, 0.1, 1, 10, and 100 μM) of dendrimer **6** (Figure 2.3). The amount of synthesized protein by macrophages, which reflects proliferation, was similar for all samples, regardless of the concentration of dendrimer in the cell

suspension. Dendrimer **6** did not change the amount of macrophage proteins; in addition, cell counting revealed no differences in cell numbers upon dendrimer addition.

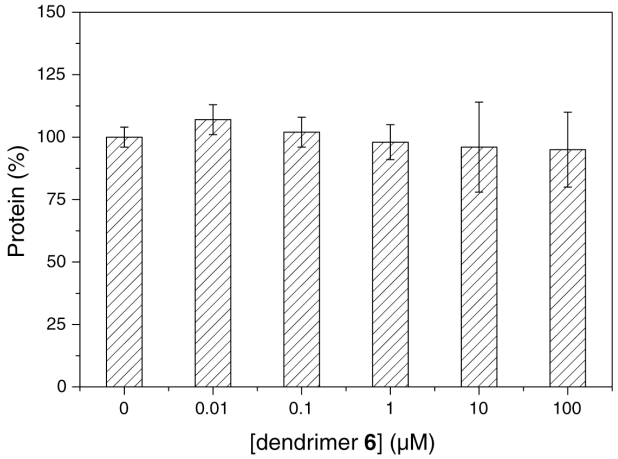


Figure 2.3 *Protein determination test using bicinchoninic acid after incubation of macrophages with dendrimer* ***6****. Error bars are standard deviations.*

The absence of toxicity for both dendrimer **4** and **6** presented here is in agreement with the model of Duncan *et al*.[22] claiming that the groups present at the periphery of the dendrimer determine the toxicity of the whole molecule: an amine-terminated dendrimer is toxic, a carboxylic acid-terminated dendrimer is non-toxic and an ethylene glycol-terminated dendrimer is non-toxic as well. As the dendrimers are designed for use in drug delivery, their host-guest properties must be investigated.

When screening for the toxicity of a compound, generally the first step is to conduct a range of *in vitro* tests, before proceeding to perform *in vivo* testing. Among the *in vitro* test systems are isolated organs, freshly isolated cells, (immortalized) cell lines, etc. Because of their ability to conduct biotransformation reactions, freshly isolated hepatocytes are often used in acute and sub-acute cytotoxicity screening. However, the current study was mainly designed to get a first indication about the toxicity of the dendrimers and hence, potential feasibility for use in biomedical applications as drug delivery agents. Clearly, to resolve a full toxicity profile of the dendrimers, a different experimental setup would be required (experimental section).

2.2.3 ENCAPSULATION OF ROSE BENGAL

The host-guest properties of dendrimer **6** were studied in buffered aqueous media at pH 7 using the anionic water-soluble xanthene dye Rose Bengal as guest molecule. Encapsulation of Rose Bengal with a dendrimer in water was mainly based on electrostatic interactions, as the tertiary amines of the dendrimer were protonated by the acids of the dye. The experiments described here were based on experiments done by Baars *et al*.[23] with the amide equivalent of dendrimer **4** (dendrimer **4'**, Figure 2.4). As dendrimer **4** and **4'** were very similar, the assumption was made that dendrimer **4** would yield similar results as dendrimer **4'**. Therefore, only encapsulation of dendrimer **6** with Rose Bengal was studied.

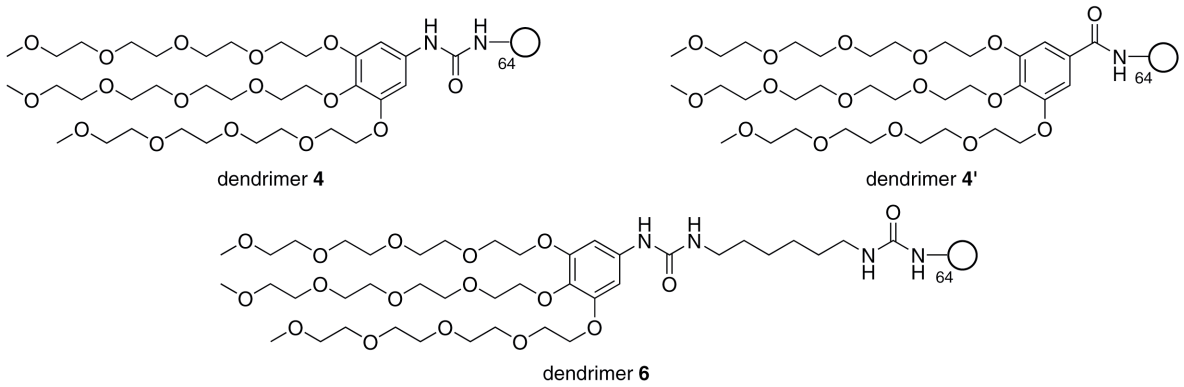


Figure 2.4 *Structural formula of dendrimer* ***4****, dendrimer* ***4'*** *and dendrimer* ***6****.*

Titration of a stock solution of dendrimer **6** to a solution of the guest yielded a bathochromic shift in the wavelength of the maximum absorption (λ_{max}) in both cases, indicative of an interaction (complex formation) between the dendrimer host and the xanthene guest (Figure 2.5, left). For comparison with the results obtained by Baars *et al.*,[23] this interaction was studied by plotting the ratio of absorptions at the wavelengths of encapsulated and free guest against the guest-host ratio (Figure 2.5, right).

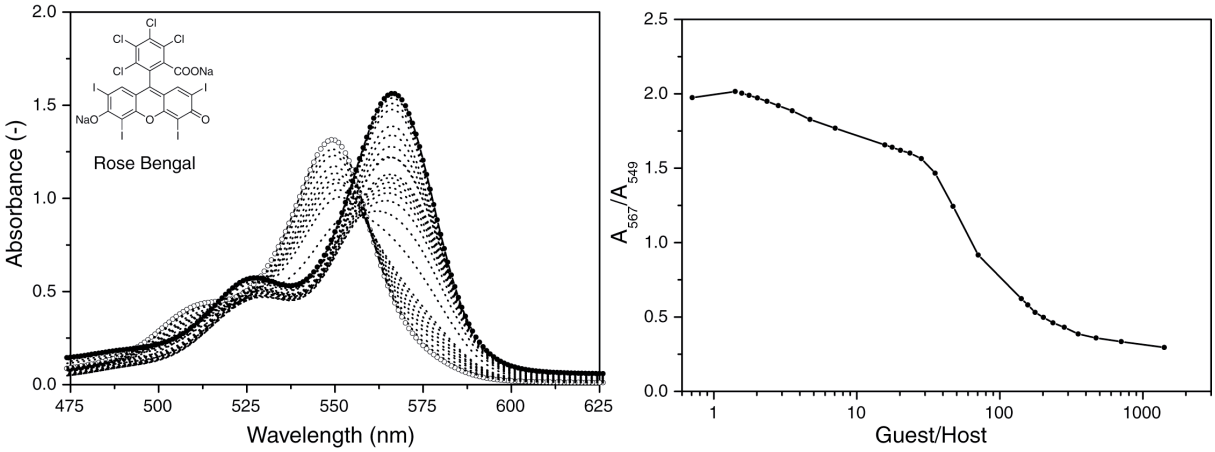


Figure 2.5 *UV/vis spectra of Rose Bengal with different concentrations of dendrimer* ***6*** *(left); and the ratio of absorptions at 567 nm and 549 nm plotted against the guest-host ratio (right).*

The UV/vis spectrum of Rose Bengal in aqueous media without host, consisted of two absorption maxima (λ_1 = 547 nm, λ_2 = 520 nm). The absorption spectrum showed changes with increasing concentration of dendrimer **6** and the ratio of the absorption maxima (λ_1/λ_2) decreased as a result of dye-dye interactions.[24,25] The λ_{max} of an encapsulated dye was determined from the UV/vis spectrum of a host-guest sample with a high guest/host ratio (GH ratio). Upon complex formation with the host, λ_{max} of the guest shifted from 549 nm to 567 nm. The graph on the right side of Figure 2.5 was obtained from the UV/vis spectra in Figure 2.5 by calculating the ratio of absorption at λ_{max} of the encapsulated guest (567 nm) and the absorption at λ_{max} of the free guest (549 nm), at each GH ratio. These results of dendrimer **6** were similar to those obtained with dendrimer **4'**,[23] indicating that encapsulation of Rose Bengal was mainly based on electrostatic interactions. To investigate in

more detail the influence of the hydrophobic domain on encapsulation, complexes of the dendrimers **4** and **6** with the cortisol were studied.

2.3. ENCAPSULATION OF CORTISOL

Cortisol was used as a model compound to investigate whether the dendrimers may be feasible as drug carriers. While the encapsulation of Rose Bengal was mainly based on electrostatic interactions; encapsulation of cortisol was based on hydrophobic interactions. The encapsulation and release of cortisol in dendrimers was studied under physiological conditions. In the next step, cortisol was modified with a urea-glycine tail (cortisol prodrug **10**) for enhancing the interactions with the dendrimers to optimize encapsulation efficiency. Finally, it was investigated whether cortisol, when loaded either directly or as a prodrug into the dendrimers still exerted glucocorticoid action in macrophages.

2.3.1 DENDRIMER – DRUG COMPLEXES

Encapsulation of cortisol with a dendrimer in water was mainly based on hydrophobic interactions. By solubilizing cortisol in the presence of dendrimer, the solubility of the drug in water was expected to increase due to hydrophobic interactions with the dendrimer. To obtain the dendrimer-drug complexes, a solution of dendrimer **4** or dendrimer **6** in chloroform was added to an excess of the drug. This solution was stirred and filtered and the obtained clear solution was evaporated. Water was added and the opalescent solution was stirred again and filtered, obtaining a clear solution of the dendrimer-drug complex in water. Due to the two filtration steps, the exact amount of cortisol present in the solution was uncertain, but as cortisol is a chiral molecule, the concentration of cortisol was approximately determined by CD spectroscopy (Figure 2.6). A UV and CD calibration curve for different concentrations of cortisol can be found in the experimental section. When no additives were present, the maximum solubility of cortisol in water was 0.77 mM.[26]

Complexes of the drug cortisol and dendrimer **4** and **6** were made in water. CD spectroscopy was used to study the enhancement in solubility of the drug. A complex of dendrimer **4** and cortisol resulted in a very small increase (1.5-fold) in solubility of the drug. Surprisingly, complex formation of cortisol with dendrimer **6** resulted in the same increase in solubility (1.5-fold increase), even though there were more possibilities for hydrophobic interactions in the later system due to the hydrophobic domain of the dendrimer. Apparently, additional hydrophobic interactions in dendrimer **6** were not sufficient for a substantial enhancement in solubility of the drug. Therefore, additional interactions will be needed to enhance the affinity between drug and carrier (section 0).

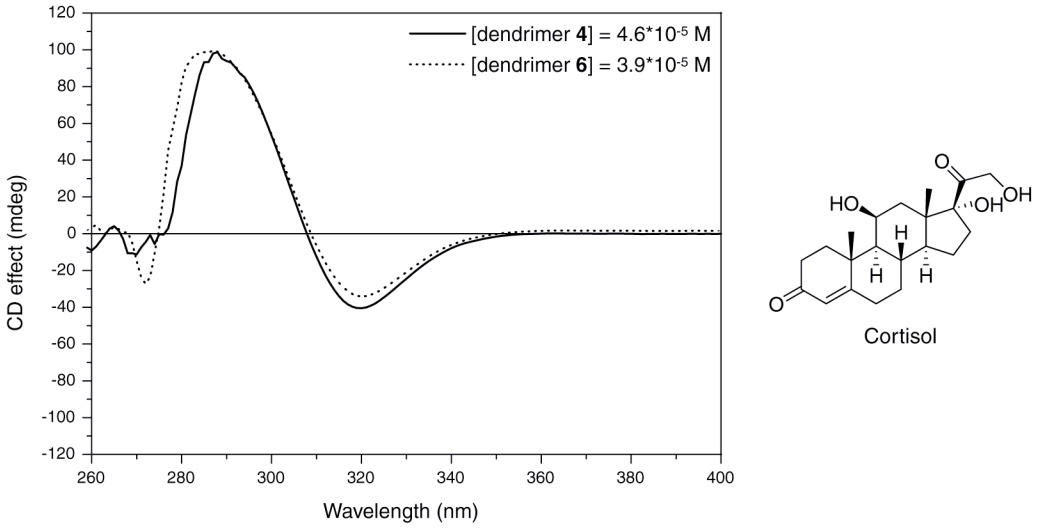


Figure 2.6 *CD measurements to determine the enhancement in solubility of cortisol in the presence of dendrimer* ***4*** *and* ***6*** *in* H_2O.

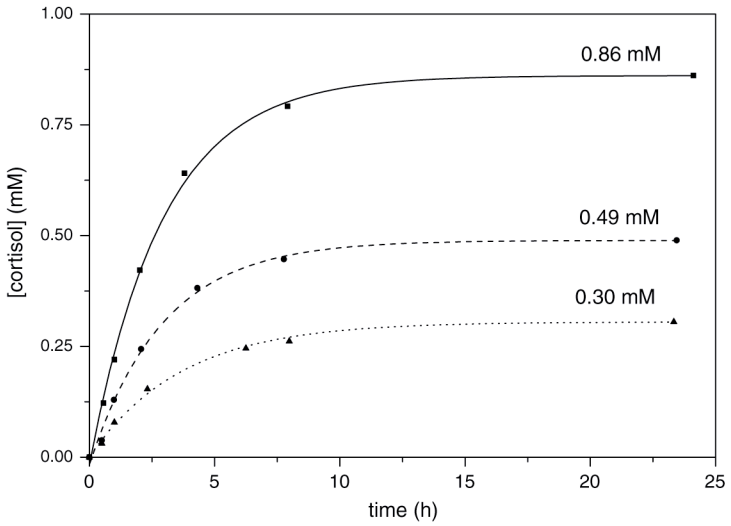


Figure 2.7 *HPLC results of dialysis experiments of a complex of dendrimer* ***6*** *with cortisol.*

First it was investigated whether the drug was still active when complexed to a dendrimer and whether the complex was dynamic, *i.e.* can the drug be released from the carrier. For a suitable drug delivery system, the dendrimer-drug complexes should be able to release the drugs again. By measuring the release of drug from the carrier with HPLC, the kinetics of the release could be examined. Dialysis of a complex of dendrimer **6** with cortisol in phosphate buffer was carried out (Figure 2.7, and experimental section) and several samples were taken over a 24 h period (solid line). Then the buffer was refreshed and dialysis was performed again. This was repeated a few times with the same complex (dashed and dotted lines). The results showed that the drug was continuously released from the carrier until a maximum concentration was reached (0.86 mM), which was comparable to the maximum concentration of cortisol in water (0.77 mM). During the second release, the concentration of cortisol reached a value lower than the first release (0.49 mM), and the third time it reached 0.30 mM. Therefore, it was concluded that the concentration of cortisol in the presence of dendrimers

was higher than the maximum solubility of cortisol in water. Also, once the dendrimer-drug complex had been formed, the dendrimer was able to release the drug again.

2.3.2 CORTISOL PRODRUG

The results with dendrimer **4** and **6** and the complex formation behavior with cortisol showed that the dendrimers could be synthesized and dendrimer-drug complexes could be formed. However, the drug load of the dendrimers, or the enhancement in drug solubility in water by adding one of the dendrimers, remained low. An attempt was made to improve the affinity of the drug for the dendrimer. Thus, a dendritic host-guest motif was used that was previously designed.[27] Through a rational design of guest molecules, a strong and selective association with the dendrimer motif could be obtained that was based on a combination of electrostatic, hydrogen bonding, and hydrophobic interactions. Previously, it was shown that this association is strong when using chloroform as solvent.[27] In order to obtain such an association between cortisol and dendrimer, the drug was modified with a urea-glycine tail, yielding the cortisol prodrug **10**. It was expected that the cortisol prodrug **10** should be able to release the active cortisol by either enzymatic or basic cleavage of the ester bond. In addition, when complexed to dendrimer **6**, the hydrophobic domain of the dendrimer might be more pronounced.

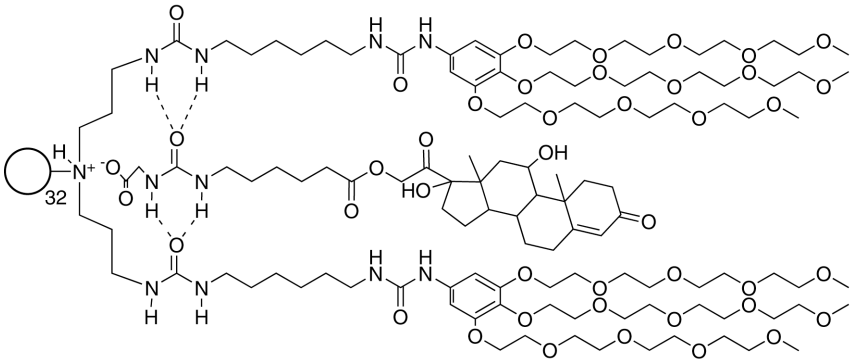
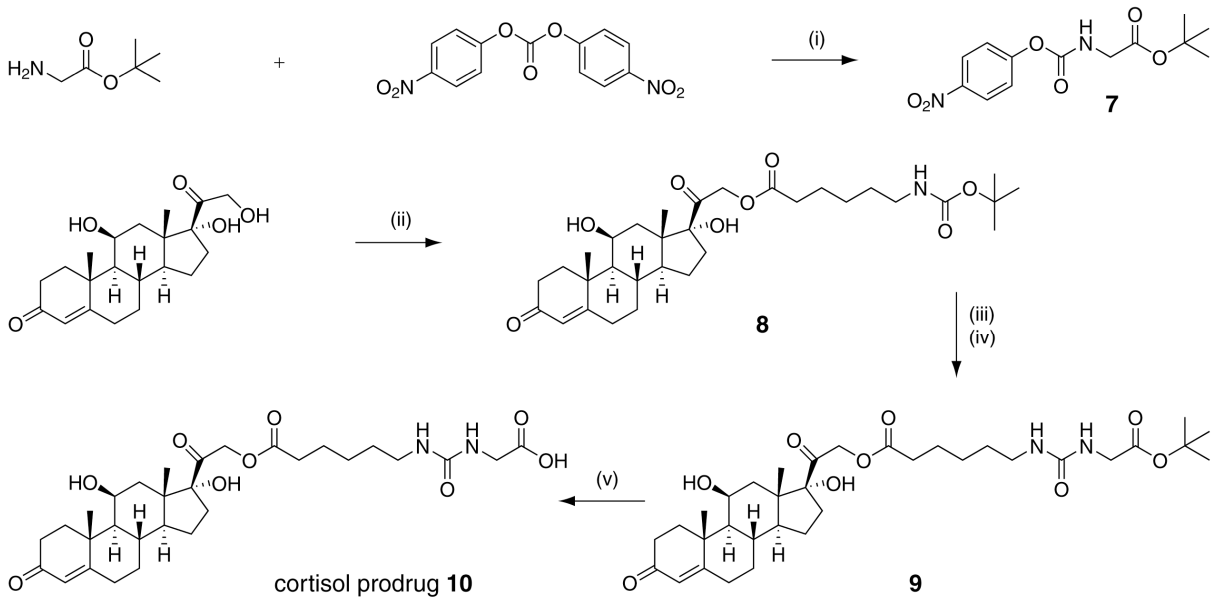


Figure 2.8 *Schematic representation of the proposed complex formation of the cortisol prodrug **10** and dendrimer **6**. Several interactions are present: electrostatic interactions between the acid of the prodrug and the tertiary amines of the dendrimer; hydrogen bonding interactions between the ureas and hydrophobic interactions between the apolar spacers. For clarity reasons, stereochemistry is not shown.*

In order to synthesize the cortisol prodrug **10**, carbamate **7** was first prepared (Scheme 2.2). The activated carbamate **7** of glycine was obtained by stirring glycine-*tert*-butyl ester and bis(4-nitrophenyl)carbonate in a dichloromethane/water mixture, yielding the product in 55% yield, upon release of *p*-nitrophenol. *Tert*-butyloxycarbonate-aminocaproic acid was coupled to the primary alcohol of cortisol via a DCC coupling method, yielding **8**. Subsequent removal of the *t*Boc-group, reaction with the activated carbamate **7** and release of *p*-nitrophenol, and subsequent hydrolysis of the *tert*-butyl ester of **9** by stirring with trifluoroacetic acid in dichloromethane, yielded the cortisol prodrug **10** (Scheme 2.2).



Scheme 2.2 *Synthesis of cortisol prodrug **10**. Reaction conditions: (i) $CHCl_3$, $NaHCO_3$, H_2O; (ii) DCC, DMAP, CH_2Cl_2,* tert-*butyloxycarbonyl-aminocaproic acid; (iii) TFA, CH_2Cl_2; (iv) **7**, Et_3N, THF; (v) TFA, CH_2Cl_2.*

2.3.3 DENDRIMER – PRODRUG COMPLEXES

Complexes of dendrimer **4** and **6** with cortisol prodrug **10** were made using the procedure as described in section 2.3.1. Based on CD spectroscopy for the prodrug **10**, a 2.6-fold increase in solubility was found for the prodrug integrated with dendrimer **4** (Figure 2.9). The complex of the drug with dendrimer **6** yielded a 5.8-fold increase in solubility of the prodrug. These data indicated that the additional interactions between dendrimer **6** and cortisol prodrug **10** (hydrogen bonding of the urea groups and acid-base interactions) account for an added solubility of the drug in water.

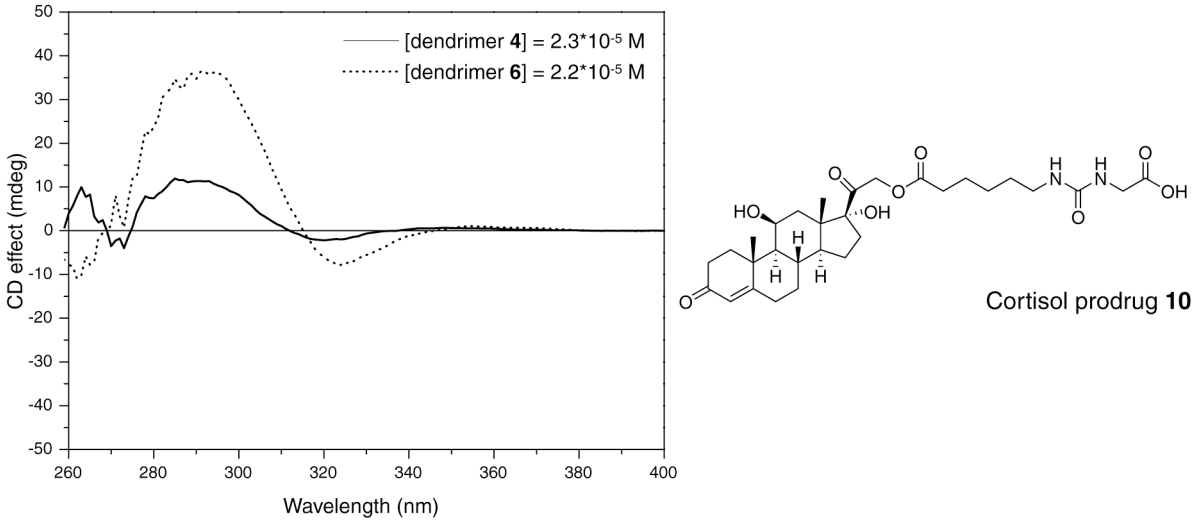


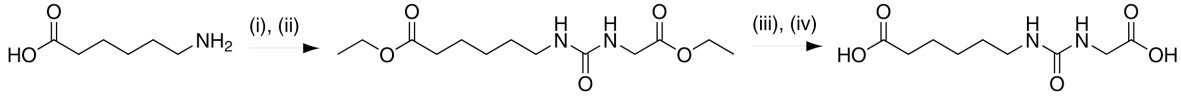
Figure 2.9 *CD measurements to determine the loading of dendrimer **4** and dendrimer **6** with cortisol prodrug **10**.*

As discussed earlier, the cortisol prodrug was the active drug designed with an esterified urea-glycine tail. It has been indicated in literature that this bond is labile, since it is under constant attack of esterases. However, the length of the tail can inhibit the esterase to cleave the ester linkage. Although this is not expected, it must be confirmed by *in vitro* tests. Therefore, the biological activity of the cortisol prodrug **10** is investigated and compared to the biological activity of the active cortisol control.

2.3.4 BIOLOGICAL ACTIVITY TESTS

The Griess assay was performed to determine the biological activity of cortisol, cortisol prodrug and complexes of cortisol prodrug with dendrimer **4**. In response to cytokines and endotoxins, the inducible nitric oxide synthetase (iNOS) in macrophages is induced, allowing the macrophages to produce a vast amount of NO radicals which is believed to contribute to the defense against bacteria. Glucocorticoids are known to suppress the induction of iNOS.[28,29] The effect of the test compounds on iNOS induction was indirectly determined by assessing the amount of nitrite in cell culture medium using the Griess assay (experimental section).

As a control, the biological activity of the urea-glycine tail **12** was tested, in order to ensure that the tail itself had no biological activity. The first step in the synthesis of **12** was conversion of the carboxylic acid of aminocaproic acid to the corresponding ethyl ester using thionylchloride and ethanol. Then the protected aminocaproic acid reacted with ethyl isocyanate acetate to yield the protected urea-glycine tail (Scheme 2.3). The protecting groups were removed by saponification. Subsequently, sodium was exchanged for hydrogen by lowering the pH, yielding the urea-glycine tail in a total yield for the four steps of 36%. The product was characterized using ^{1}H, COSY, and ^{13}C NMR spectroscopy measurements and elemental analysis.



Scheme 2.3 *Synthesis of urea-glycine tail **12**. Reaction conditions: (i) thionyl chloride, ethanol; (ii) CH_2Cl_2, ethyl isocyanate acetate; (iii) NaOH, THF, water; (iv) HCl, water.*

After synthesis of **12**, its potency to inhibit nitrite production was tested (Figure 2.10, right). The tail **12** showed no significant biological activity, meaning that if the biological activity of cortisol prodrug **10** was triggered by removal of the tail by esterases, the removed tail played no part in the inhibition of nitrite production. In that case, the biological activity as displayed in Figure 2.10 could be contributed to the formation of the original cortisol by the action of esterases. Also dendrimer **4** was tested and showed no significant biological activity.

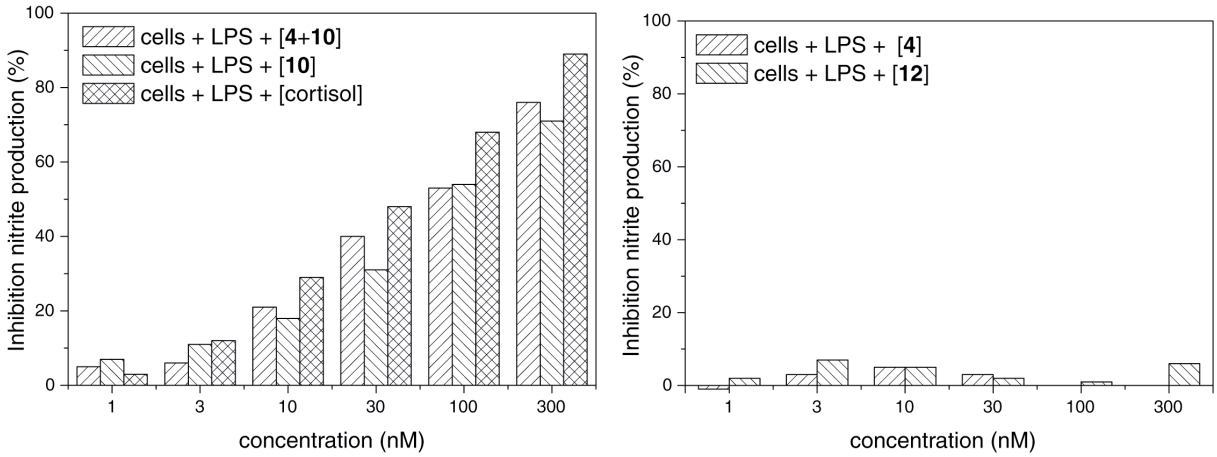


Figure 2.10 *Griess Assay showing the inhibition of nitrite production using cortisol, cortisol prodrug **10** and a complex of the prodrug and dendrimer **4**. On the left the inhibition of nitrite production by only the dendrimer or **12** is shown (controls).*

The results of the dendrimer-drug complexes showed that cortisol inhibits lipopolysaccharide-induced (LPS-induced) NO formation in a concentration dependent manner (Figure 2.10, left). A similar pattern for cortisol was also observed for the cortisol prodrug **10** and dendrimer **4** carrying the prodrug. From Figure 2.10, it could be concluded that the prodrug and dendrimer **4** carrying the prodrug were approximately equal potency — on a molar basis — as cortisol itself. Hence, introduction of the ester group in the side chain of the cortisol did not seem to greatly influence the biological activity of the drug. Similarly, complexing the prodrug with the dendrimer did not appear to reduce the biological potency of the drug. However, as shown by CD spectroscopy (section 2.3.3), the local concentration of prodrug **10** in dendrimer **4** was higher (2.6×) compared to the maximum concentration of cortisol in water. This suggested that the potency of the modified cortisol was in fact slightly reduced through complexation of the molecule with the dendrimer, although for an exact quantification of the potencies, IC_{50} values should be calculated. Nevertheless, considerable activity was still observed with the dendrimer complex. The reduction of nitrite produced by the prodrug or the dendrimer-prodrug complex seemed to be specific since no effect of the tail **12** or the dendrimer itself was observed (Figure 2.10, right). In addition, the dendrimer did not have an effect on the amount of nitrite produced by the macrophages in the absence of LPS, indicating that the dendrimer had no pro-inflammatory effect.

The graph in Figure 2.10 showed that cortisol displayed glucocorticoid activity, since it inhibited the production of nitrite of macrophages induced by LPS upon increasing concentrations of cortisol. The biological activity of the cortisol prodrug was in the same order of magnitude as cortisol. It was expected that the ester of cortisol prodrug **10** was under constant attack of esterases, thereby breaking the ester bond and releasing the intact cortisol. The biological activity of the cortisol prodrug **10** was in agreement with this assumption, as the prodrug inhibited the nitrite production. In addition, a complex of

dendrimer **4** and the cortisol prodrug **10** was incubated with cells and LPS and showed comparable biological activity. Thus, it was concluded that a complex of dendrimer **4** and cortisol prodrug **10**, the prodrug itself and cortisol all show similar biological activities. Since it was not determined whether cortisol was actually produced in these incubations, an alternative explanation may be that the prodrug itself displays a similar activity as the cortisol.

2.4. CONCLUSIONS

A convenient methodology was developed for the synthesis of water-soluble dendrimers possessing a hydrophobic domain. The dendrimers were not toxic to rat macrophages and showed no induced cell proliferation. They were able to form complexes with the dye Rose Bengal, through electrostatic interactions. The drug cortisol was used as a model system to study the encapsulation of drugs in two different dendrimers, a dendrimer with and without a hydrophobic domain. To strengthen the complex formation of dendrimer and drug, cortisol was modified with a urea-glycine tail in order to have a combination of multiple interactions (electrostatic, hydrophobic and hydrogen bonding) between dendrimer and cortisol. It was found that the most efficient encapsulation occurred when both host and guest possessed hydrophobic domains resulting in an almost 6-fold increase in solubility. Both cortisol and the cortisol prodrug, as well as dendrimer-drug complexes, showed similar biological activities, indicating the release of active cortisol from the carrier and degradation of the ester bond in case of the prodrug.

2.5. EXPERIMENTAL SECTION

General methods

Unless stated otherwise, all solvents (*p.a.* quality) were obtained from commercial sources and used as received. MilliQ water is demineralized, distilled and filtered water, made using a Super-Q Plus of Millipore. Water was demineralized prior to use. Dichloromethane was obtained by distillation from P_2O_5. Diisopropylethylamine was distilled from ninhydrin and KOH before being used. Aminocaproic acid (Janssen Chimica), di-*tert*-butyl dicarbonate (Acros), bis(4-nitrophenyl)carbonate (Aldrich), cortisol (Sigma), LPS (Sigma), sulfanilaminde (Sigma), N-(1-naphtyl)-ethylenediamine (Sigma), $NaNO_2$ (Merck), thionylchloride (Merck), aminocaproic acid (Janssen Chimica) and ethyl isocyanate acetate (Aldrich) were used as received. FK-12 medium (sigma), fetal bovine serum (FBS, Sigma), triton X-100 (Sigma), acetaminophen (Sigma), *tert*-butyl hydroxyperoxide (Simga) NADH (Merck), DMSO (ICN), and sodium pyruvate (Roche) were of the highest quality available and used as received. Amine-terminated poly(propylene imine) dendrimers DAB-*dendr*-$(NH_2)_{64}$ were kindly provided by SymoChem (Eindhoven, The Netherlands) and dried prior to use. Di-*tert*-butyl tricarbonate was prepared according to the procedure described by Peerlings *et al*.[30]

Standard ^1H and ^{13}C NMR spectroscopy experiments were performed on a Varian Gemini-2000 300 MHz spectrometer, a Varian Mercury Vx 400 MHz spectrometer, and a Varian Unity Inova 500 MHz spectrometer at 298 K. Chemical shifts are reported in parts per million relative to tetramethylsilane (TMS). NMR spectroscopy solvents $CDCl_3$, and CD_3COCD_3 were purchased from Cambridge Isotope Laboratories. Infrared spectra were recorded at 298 K on a Perkin-Elmer 1605 FT-IR spectrophotometer. ES-QTOF-MS experiments were recorded on a Q-TOF Ultima GLOBAL mass spectrometer (Micromass, Manchester, U.K.). MALDI-TOF MS spectra were recorded on a Perspective DE Voyager spectrometer utilizing a α-cyano-4-hydroxycinnamic acid matrix. The presence of amine and urea groups was verified using ninhydrin and TDM[31]. Fluorescence excitation and emission experiments were performed on a Perkin-Elmer Luminescence spectrometer LS 50B. CD spectroscopy measurements were performed on a JASCO J-600 instrument. The UV-Vis spectra were recorded on a Perkin Elmer Lambda 900 spectrophotometer. Dialysis experiments were carried out on a Spectra/Por dialyzer of Spectrum, with accompanying molecular porous regenerated cellulose dialysis membranes.

*Toluene-4-sulfonic acid 2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethyl ester (**1**)*

A solution of tetra(ethylene glycol) (25 g, 1 eq, 0.12 mol) in 40 ml of THF was added to a solution of sodium hydroxide (40 g, 1.6 eq, 0.19 mol) in 40 ml of distilled water at RT. The mixture was stirred and cooled to -5 °C. A solution of toluene-4-sulfonyl chloride (22.9 g, 1 eq, 120 mmol) in 40 ml of THF was added dropwise to the reaction mixture at -5 °C. The reaction was stirred at -5 °C for 3.5 h and was allowed to warm up to room temperature overnight. The mixture was poured into ice and extracted with dichloromethane. The organic layers were washed with water and brine and subsequently dried over sodium sulfate. After filtration and evaporation, a colorless oil was obtained in 93% yield, that was used as such in the next step. ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 2.35 (s, 3H, CH_3) 3.4 (s, 3H, CH_3) 3.42-3.8 (m, 14H, 7×CH_2) 4.2 (t, 2H, CH_2) 7.4 (d, 2H, 2×CH arom) 7.8 (d, 2H, 2×CH arom); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 145.0 (C_q arom), 133.2 (C_q arom), 130.0 (2×CH arom), 128.2 (2×CH arom), 72.1 (CH_2), 70.9 (CH_2), 70.8 (CH_2), 70.7 (CH_2), 69.4 (2×CH_2), 68.9 (2×CH_2), 59.2 (CH_3), 21.8 (CH_3).

*3,4,5-Tris-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-benzoic acid (**2**)*

Methyl gallate (5.2 g, 1 eqv, 28 mmol) was dissolved in 200 ml of 4-methyl-pentan-2-one under argon. Compound **1** was added dropwise and the reaction mixture was refluxed overnight. The reaction was filtrated and the solvent was removed *in vacuo*. The obtained product was dissolved in dichloromethane and washed several times with water. The organic layers were combined, dried over sodium sulfate and the solvent was evaporated *in vacuo*. The obtained oil was purified by column chromatography (dimethoxyethane: heptane, 1:1), yielding 83% of colorless oil. A solution of the obtained methyl gallate derivative (5.3 g, 1 eq, 7 mmol) in 5 ml of methanol was added to a solution of sodium hydroxide (0.42 g, 1.5 eq, 10 mmol) in 5 ml of water and the mixture was stirred overnight.

Hydrochloric acid was added to neutralize the mixture and an extraction with dichloromethane was performed. The organic layers were combined, dried over sodium sulfate and the solvent was evaporated *in vacuo*, yielding the product as colorless oil in 93% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 3.30 (s, 3H, CH_3), 3.33 (s, 6H, 2×CH_3), 3.42-3.54 (m, 6H, 3×CH_2), 3.54-3.68 (m, 30H, 15×CH_2), 3.69-3.81 (m, 6H, 3×CH_2), 4.15 (m, 6H, 3×CH_2), 7.19 (s, 2H, 2×CH); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) 169.8, 152.3, 143.1, 124.6, 109.7, 72.5, 71.9, 70.8, 70.6, 70.4, 69.7, 68.9, 59.0; IR (FT) ν (cm^{-1}) 1714 (C=O).

*3,4,5-Tris-(2-{2-[2-(2-methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-phenylisocynanate (**3**)*

Compound **2** (3.3 g, 1 eq, 4.5 mmol) and triethylamine (1.1 ml, 2 eq. 8 mmol) were dissolved in 20 ml of distilled THF. The reaction mixture was cooled to 0 °C and a solution of ethylchloroformate (0.77 ml, 2 eq, 8 mmol) in 3 ml of THF was added dropwise. The reaction was stirred overnight and monitored with IR spectroscopy. The obtained slurry was added under argon to a cooled solution (0°C) of sodium azide (5.9 g, 20 eq, 90 mmol) in 30 ml of distilled water. The reaction was stirred for 2 h and monitored by IR spectroscopy. An extraction was performed with dichloromethane and the combined organic layers were dried over sodium sulfate. After filtration, the solvent was removed *in vacuo*. The obtained oil was dissolved in 25 ml of distilled dioxane and the solution was refluxed for 2.5 h to perform a Curtius rearrangement. After completion (monitored by IR spectroscopy) the solution was cooled to 90 °C. The solvents were evaporated *in vacuo* yielding the product as yellow oil. IR (FT) ν (cm^{-1}) isocyanate: 2266 (NCO).

*Dendrimer **4***

The poly(propylene imine) dendrimer (G5) was stripped with toluene several times to remove water. To a solution of 78 mg (1 eq, 0.011 mmol) of the dried dendrimer in 5 mL of chloroform, 0.52 g (64 eq, 0.7 mmol) of **3** was added. The reaction was stirred and monitored by IR spectroscopy. After completion, the solvent was removed to yield the product as colorless oil in 81% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 1.40-1.80 (br, 636H) 2.10-2.60 (m, 1152H) 3.36 (br s, 576 H) 3.51-3.9 (m, 2688H), 4.15 (m, 384H) 6.6 (br s 128H); ^{13}C NMR (100 MHz, $CDCl_3$/$(CD_3)_2CO$) δ (ppm) 152.1, 71.9, 71.4, 70.2, 70.1, 70.0, 69.9, 69.2, 68.4, 58.3, 58.2; IR (FT) ν (cm^{-1}) 1662.8 (C=O), 2870.5 (C-H sat), 3331.8 (NH stretch); Elemental analysis: C 55.92 % (calc. 56.35), H 8.74 % (calc. 8.63), N 4.72 % (calc. 4.84).

*3,4,5-Tris(2,5,8,11-tetraoxatridecan-13-yloxy)benzenamine (**5**)*

Isocyanate **3** was dissolved in dioxane and added dropwise to a solution of tetrabutylammonium hydroxide (40% in water, 2.2 eq) in dioxane at 90 °C. The reaction was stirred for 15 minutes and subsequently cooled to room temperature. The solvents were evaporated *in vacuo* and an extraction was carried out with dichloromethane and water. The organic layers were combined and dried over sodium sulfate. The solvent was removed *in vacuo* yielding the amine **5** as yellow oil, which was purified by column chromatography using 2% of methanol in CH_2Cl_2 yielding the product in 74%. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 3.36 (s, 9 H, OCH_3), 3.51-3.82 (m, 44 H, OCH_2+NH_2), 5.97 (s, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ (ppm) 153.1 ($2C_q$, ar), 142.9 (C_q, ar-NH_2), 131.0 (C_q, ar), 95.5 (CH, ar), 95.3 (CH, ar), 72.4-68.5 (m, OCH_2), 59.0 (s, 3C, OCH_3).

*Dendrimer **6***

Amine **5** (2.6 g, 1 eq, 3.7 mmol) was dissolved in toluene and added dropwise to hexamethylene diisocyanate (17.6 ml, 30 eqv, 110 mmol) under argon. The reaction mixture was stirred for an additional 30 minutes and the product was precipitated three times in heptane. A white sticky solid was obtained. The poly(propylene imine) dendrimer (G5) was stripped with toluene several times to remove water. To a solution of dried dendrimer (0.15 g, 1 eq, 0.021 mmol) in toluene, the mono-isocyanate (1 g, 64 eq, 1.13 mmol) dissolved in toluene was added dropwise. The product was purified using precipitation in heptane and extensive dialysis followed by freeze-drying, resulting in a colorless oil (85% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 1.05-1.6 (br, 1404H) 2.38 (br, 1408H) 3.3 (br s, 576 H) 3.42-3.9 (m, 2688), 4.15 (m, 384H) 6.6 (s, 128H). Elemental Analysis: C 56.51% (calc. 56.75), H 8.88% (calc. 8.81), N 6.90% (calc. 7.02).

Cell culture conditions

Rat macrophages (NR8383) were obtained from the ATCC (American Type Culture Collection) and were cultured in FK-12 medium (Gibco, BRL), supplemented with 10 v/v % heat inactivated foetal calf

serum (Sigma), L-glutamine (292 mg/L) and the antibiotics penicillin (at 10.000 U/L) and streptomycin (at 100 mg/L). The cells were grown in 75 cm^2 flasks in a humidified atmosphere containing 5% CO_2 at 37 ^oC. Medium was replaced every two days and the cells were split if they reached a density exceeding 8 million cells per flask. All procedures with the cells were conducted under sterile conditions. Cells were regularly inspected using a Zeiss Axioscope microscope and counted using a Buerker chamber.

Cell toxicity tests

For conducting toxicity tests (MTT test, Trypan blue, or NADH permeation tests), cells were seeded (10^5 cells per well) in 24-well plates in the same medium as described above, but with reduced (2,5 v/v %) foetal calf serum, to arrest cell growth. Stock solutions of the dendrimers were prepared in complete culture medium with reduced FCS and sterilized by filtration (0.2 µm). Dendrimers were added to the cells to obtain final concentrations of 0, 0.3, 1.0, 3.0, 10 and 30 µM for dendrimer **4** or 0, 0.01, 0.1, 1.0, 10 and 100 µM for dendrimer **6**. The final volume was 1 ml per well. Blank incubations (complete incubations but without cells) were included. All incubations were conducted at least in triplicate.

Following 48 h of incubation in a humidified atmosphere containing 5% CO_2 at 37 ^oC, the cells and medium were harvested for performing the Trypan blue assay, NADH permeation or MTT tests.

The Trypan blue test[32] and NADH permeation test[33,34] reflect the integrity of the cellular membrane. If the cell membrane is leaky, the Trypan blue stain will be able to penetrate the cell interior, yielding a cell with a blue appearance that can be visualized under a light microscope. By counting the number of blue stained cells and the total number of cells, the % age of damaged cells can be determined. Briefly, 25 µL aliquots of the cell suspension were mixed with 30 µL of a 5.2 mM Trypan blue solution in PBS (phosphate buffered saline, i.e. 10 mM of o-phosphoric acid and 150 mM NaCl dissolved in milliQ water and brought to pH=7.4 with KOH), allowed to stand for 1 minute, after which an aliquot of the cells were applied to a Buerker chamber to count total and blue cell numbers using a Zeiss Axioscope microscope and % viability determined as 100- 100x stained/total number of cells.

In the NADH permeation assay,[33,34] the activity of lactate dehydrogenase activity is determined by spectrophotometry in an aliquot of cell suspension by adding substrate (sodium pyruvate) and cofactor (NADH). Given the fact that the LDH is an intracellular enzyme, this activity represents the LDH that has leaked out of the cells. By subsequently solubilizing the cells using Triton X-100 as detergent, the total cellular LDH activity can be determined. To measure NADH permeation, 100 µL of cell suspension and 900 µL of a solution of 0.2 mg/ml NADH and 0.6 mg/ml sodium pyruvate in PBS were mixed in a cuvette to monitor the decrease of absorbance at 340 nm in a Philips PU8740 UV/VIS spectrophotometer for 40 seconds. Subsequently, 100 µL of a 10 % solution of Triton X-100 in PBS was added to the cuvette, following immediate mixing to solubilize the cells. Again, the decrease of absorbance at 340 nm was assessed, and corrected for the volume increase. Appropriate blanks were included in the assay and subtracted from obtained values. Viability (%) was calculated as 100 - 100 x (A_{340} decrease without/ A_{340} decrease with Triton). The Triton X-100 did not change the LDH activity released into the medium by dead cells (as assessed in medium that was separated from the cells by centrifugation).

In the MTT test[35] cell redox activity is screened for. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide] is reduced by membrane bound (mitochondrial and microsomal) enzymes to produce non water soluble formazan that can quantitated by spectrophotometry. The read out correlates with the amount of patent cellular electron carriers (being highly vulnerable if cellular function is compromised) and thus indirectly indicates the cellular status. For the dendrimers, the MTT-test was performed by incubating a mixture of 150 µL cell suspension, 150 µL of complete FK-12-medium and 60 µL MTT (4.8 mM in PBS) for 3.5 hours at 37 ^oC. The produced formazan was dissolved by adding 900 µL of acetone and mixing for 10 min. Subsequently the absorbance of the samples at 570 nm was measured using a Philips PU8740 UV/VIS spectrophotometer. Formazan production was standardized for the amount of cellular protein that was added. Obtained values for each test compound (dendrimer) were standardized for the cells incubated without the test-compound (=value set at 100%).

Cellular protein concentrations were determined using the Bicinchoninic acid (BCA) assay as described.[36] For this, the harvested cells were centrifuged at 300 g, and cell pellets washed twice by resuspension in PBS and centrifugation.

Characterization of host-guest properties
UV/vis titration with Rose Bengal.[23] Stock solutions of $2.5*10^{-5}$ M dendrimer in PBS were added in aliquots of 1-1000 µL to a $1.25*10^{-5}$ M solution of Bengal Rose (2 ml in a quartz cell). UV/Vis spectra were recorded after mixing and corrected for dilution.

Dendrimer-drug complexes
For a typical complex formation of dendrimer and drug, in a test tube the dendrimer **6** (10 mg, 0.18 µmol, 1 eq) was dissolved in 1 mL of chloroform and guest cortisol (2.13 mg, 5.88 µmol, 32 eq) was added, this suspension was stirred for a few minutes. The suspension was filtered over a micro filter (0.2 µm), resulting in a clear solution. Chloroform was evaporated by nitrogen flow to obtain a film. Water or buffer was added and the mixture was stirred for a few minutes until the film was completely dissolved. The suspension was filtered over a micro filter (0,2 µm), resulting in a clear solution in water. This solution was used for further experiments.

Determining drug load by CD and UV spectroscopy
For the UV/vis experiments with cortisol, all samples were measured in quartz cells. A calibration curve of cortisol was made, which reflects the absorption at 248 nm vs. the concentration of cortisol and a straight line was obtained.

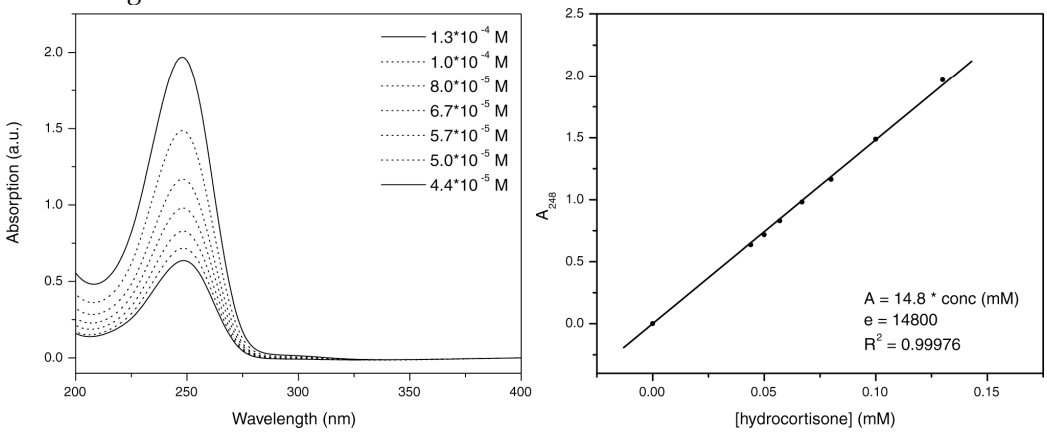


Figure 2.11 *UV/vis calibration curve for Rose Bengal.*

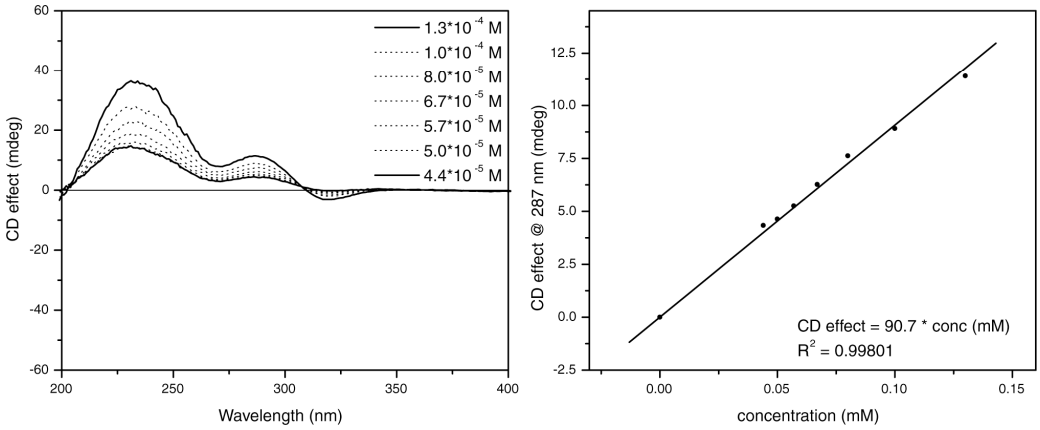


Figure 2.12 *CD calibration curve for cortisol.*

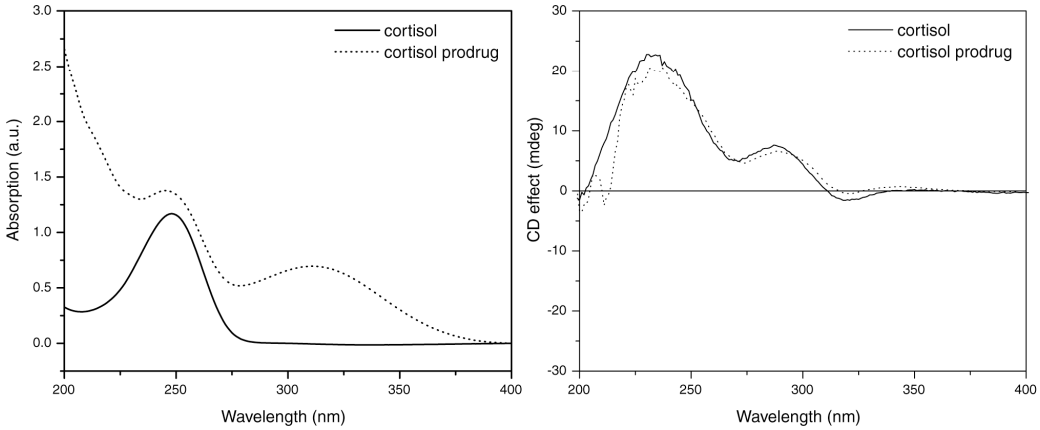


Figure 2.13 *UV/vis and CD spectra of cortisol and the cortisol prodrug **10** in water.*

For the CD experiments with cortisol, all samples were measured in quartz cells. A calibration curve of cortisol was made, which reflects the CD effect at 287 nm vs. the concentration of cortisol, a straight line is obtained. Subsequently, the dendrimer-drug complexes are measured and the obtained value is related to the calibration curve. The amount of dendrimer is known, as we assume that no dendrimer is lost during the filtration steps in the complex formation. In this way, the amount of cortisol could be determined and with this, the guest-host ratio.

Dialysis experiments

The dialysis cell used in this experiment consists of two compartments separated by a membrane disk (Figure 2.14). The dendrimer-drug complex in a phosphate buffer of pH 7 is put in the left compartment of a dialysis cell. In the right compartment buffer was put into. Molecular weight cut off of the used membrane disks is 12000-14000. The membrane disks are washed and soaked in water for a few minutes and directly used for dialysis. Samples were taken from the right compartment seven times during dialysis. After 24 hours, the right compartment was emptied, new buffer was put in, and dialysis was started again. This was repeated a few times. Samples were analyzed by HPLC with a UV detector (238 nm). The area under the cortisol peak shows a linear correlation with the concentration of cortisol. Control experiments show that cortisol did not stick to the membrane. In a second control experiment, the absorption capacity of the dendrimer was examined by adding the dendrimer on one side of the membrane to a saturated solution of cortisol in PBS. This was dialyzed against a saturated solution of cortisol in PBS. Unfortunately, the dendrimer is not able to absorb the drug molecule from the solution.

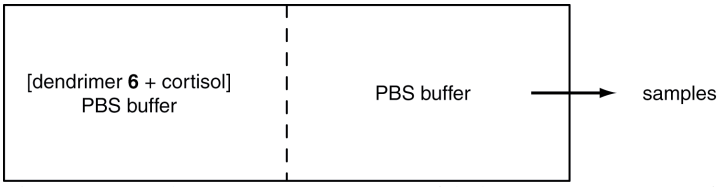


Figure 2.14 *Schematic representation of dialysis experiments performed.*

t*Boc-aminocaproic acid*

NaOH (23 mL, 1 M solution in water) was added to a solution of aminocaproic acid (1.50 g, 11.4 mmol) in a 1:1 mixture of dioxane and water (11 mL). After the addition of di-*tert*-butyl dicarbonate (2.49 g, 11.4 mmol) in 11.4 mL of dioxane, the pH was checked and kept higher that 8 by adding a 1 M solution of NaOH. The reaction was monitored by TLC and stirred overnight. After concentration under reduced pressure and addition of ethyl acetate and a 1 M solution of $KHSO_4$, the solution was washed with 1 M $KHSO_4$. After drying over $MgSO_4$, ethyl acetate was removed under educed pressure. Addition of $CHCl_3$, and subsequent evaporation under reduced pressure (up to 1 mbar) the product was obtained in 92% yield (2.44 g). ^{1}H NMR ($CDCl_3$) δ = 5.47 (s, 1H, COOH), 4.55 (m, 1H, NH), 3.11 (m, 2H, CH_2), 2.35 (t, *J*=14.65 Hz, 2H, CH_2), 1.65 (quintet, *J*=30.02 Hz, 2H, CH_2), 1.50 (quintet, *J*=29.29 Hz, 2H, CH_2), 1.44 (s, 9H, 3×CH_3), 1.37 (quintet, *J*=30.02 Hz, 2H, CH_2). ^{13}C NMR (DMSO) δ =

174.5 (COOH), 156.6 (CO), 77.3 (C_q), 33.7 (CH_2), 29.3 (CH_2), 28.3 (3C, CH_3), 25.9 (CH_2), 24.3 (CH_2).[37] Rf value = 0.51 in (CH_2Cl_2:MeOH:AcOH) (89:10:1)

*2-((8S,9S,10R,11R,13S,14S,17R)-11,17-dihydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl 6-(*tert*-butoxycarbonylamino)hexanoate (**8**)*
Cortisol (1.1 g, 1 eq, 3 mmol), 6-*tert*-Butoxycarbonylamino-caproic acid (0.7 g, 1 eq, 3 mmol), *N,N'*-dicyclohexylcarbodiimide (DCC, 0.69 g, 1.1 eq, 3.3 mmol), and 4-dimethylaminopyridine (DMAP, 0.07 g, 0.2 eq, 0.6 mmol), were dissolved in 50 ml of dried CH_2Cl_2 and the reaction mixture was stirred overnight. The suspension was filtered in order to remove dicyclohexylurea (DCU) and the solvent was evaporated *in vacuo*. The crude product was dissolved in ethyl acetate and washed subsequently with 1 M potassium bisulphate, 5% sodium bicarbonate and brine. The organic layers were combined and dried over sodium sulfate and the solvent was evaporated *in vacuo*. Column chromatography was performed using 2% of methanol in CH_2Cl_2, yielding the product in 68% yield. ^{1}H NMR (300 MHz, DMSO) δ (ppm) 0.7-2.6 (crowded; 23H of cortisol, 8H of C6-tail, 9H of tBu-group), 2.88 (q, 2H, CH_2), 4.27 (s, 1H, OH), 4.25 (d, 1H, CH), 4.7 (dd, 1H, CH_2), 5.1 (dd, 1H, CH_2), 5.40 (s, 1H, OH), 5.56 (s, 1H, CH), 6.76 (t, 1H, NH). ^{13}C NMR (75 MHz, DMSO) δ = 205.4 (C-20), 198.1 (C-3), 172.4 (C-22), 172.3 (C-5), 155.5 (C-29), 121.5 (C-4), 88.7 (C-17), 67.4 (C-21), 66.4 (C-11), 55.6 (C-9), 51.6 (C-14), 46.8 (C-13), 39.7 (C-12), 38.9 (C-10), 34.1 (C-1), 33.5 (C-2), 33.4 ($C(CH_3)_3$), 33.2 (C-23), 33.1 (C-16), 32.8 (C-6), 31.4 (C-7), 31.1 (C-8), 29.2 (C-26), 28.3 ($C(CH_3)_3$), 25.7 (C-25), 24.2 (C-24), 23.3 (C-15), 20.5 (C-19), 16.6 (C-18).[37] ESI-MS: 598 ($M+Na^+$), 1173 ($M+H^++Na^+$). R_f value = 0.18 in (EtOAc:Hexane) (1:1).

(4-Nitro-phenoxycarbonylamino)-acetic acid tert*-butyl ester (**7**)*
To a solution of glycine *tert*-butyl ester hydrochloride (Fluka 99+%, 1.0 g, 1 eq, 5.96 mmol) and bis-(4-nitrophenyl) carbonate (Aldrich 99+%, 1.8 g, 1 eq, 5.96 mmol) in 20 ml of dichloromethane was added a solution of 15 ml of sodium bicarbonate (saturated in water) and 15 of water, upon addition a green color is observed. After stirring for 21 hours, layers were separated and the organic layer was washed three times with a saturated solution of sodium carbonate, and one time with brine. The combined organic layers were dried over sodium sulfate and the crude product was obtained by evaporation of the solvents *in vacuo*. Recrystallization was performed from a 4:1 mixture of hexane and ethyl acetate, resulting in white crystals, which were dried *in vacuo* at 40 °C. Yield is 55%. ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 1.51 (s, 9H, 3×CH_3), 3.9 (d, 2H, CH_2), 5.68 (s, 1H, NH), 7.3 (dd, 2H, 2×CH), 8.2 (dd, 2H, 2×CH); ^{13}C NMR (125 MHz, $CDCl_3$) δ = 168.6 (COO), 155.8 (C_q(Ph)), 153.2 (CONH), 144.9 (NO_2C_q(Ph)), 125.2 (2C, Ph), 122.1 (2C, Ph), 83.0 ($C(CH_3)_3$), 43.5 ($NHCH_2$), 28.1 ($C(CH_3)_3$). Elemental analysis; Calculated: C 52.70%, H 5.44%, N 9.45%. Found: C 53.0%, H 5.33%, N 9.71%. IR (FT) ν (cm^{-1}) 1717 (C=O). Mp = 98-99 °C.

*2-((8S,9S,10R,11R,13S,14S,17R)-11,17-dihydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethyl 6-(3-(2-*tert*-butoxy-2-oxoethyl)ureido)hexanoate (**9**)*
Compound **8** (4.13 g, 7.17 mmol) was dissolved in 22 mL CH_2Cl_2, 7 mL of TFA was added slowly and the reaction was monitored with TLC. After 30 minutes, the solvent was evaporated *in vacuo* and the product was stripped with THF several times to remove excess of TFA. The product (1.7 g, 1 eq, 1 mmol) was dissolved in DMF, compound **7** (2.16 g, 4.5 mmol, 1 eq) and diisopropylamine (1.3 ml, 9 mmol, 2 eq) were added and the reaction was stirred overnight. The pH of the reaction mixture was checked regularly and the solution was kept basic by addition of DIPEA. All solvents were evaporated under reduced pressure and ethyl acetate was added. The solution was washed subsequently with 1 M of a potassium bisulfate solution, water, and 5% sodium bicarbonate solution. The organic layers were dried over sodium sulfate and the solvent was evaporated *in vacuo*. Precipitation in diethyl ether was performed to yield **9** as a white solid (1.61 g, 35% yield). ^{1}H NMR (300 MHz, DMSO) δ (ppm) 0.7-2.6 (crowded; 23H of cortisol, 8H of C6-tail, 9H of tBu-group), 3.03 (q, 2H, CH_2), 3.7 (d, 2H, CH_2), 4.3 (s, 1H, OH), 4.4 (d, 1H, CH), 4.8 (dd, 1H, CH_2), 5.1 (dd, 1H, CH_2), 5.40 (s, 1H, OH), 5. 6 (s, 1H, CH), 6.0 (t, 1H, NH), 6.1 (t, 1H, NH). ^{13}C NMR (100 MHz, DMSO) δ = 205.3 (C-20), 198.1 (C-3), 172.4 (C-22), 172.4 (C-5), 170.4 (C-32), 157.9 (C-29), 121.5 (C-4), 88.7 (C-17), 80.2 ($C(CH_3)_3$), 67.4 (C-21), 66.3 (C-11), 55.6 (C-9), 51.6 (C-14), 46.8 (C-13), 42.1 (C-31), 39.0 (C-12), 38.9 (C-10), 34.1 (C-1), 33.5 (C-23), 33.2 (C-16), 33.1

(C-2), 32.8 (C-6), 31.4 (C-7), 31.1 (C-8), 29.7 (C-26), 27.8 (C(CH_3)$_3$)), 25.8 (C-25), 24.2 (C-24), 23.3 (C-15), 20.5 (C-19), 16.6 (C-18).[37]

*2-(3-(6-(2-((8S,9S,10R,11R,13S,14S,17R)-11,17-dihydroxy-10,13-dimethyl-3-oxo-2,3,6,7,8,9,10,11,12,13,14, 15,16,17-tetradecahydro-1H-cyclopenta[a]phenanthren-17-yl)-2-oxoethoxy)-6-oxohexyl)ureido)acetic acid (cortisol prodrug **10**)*

Compound **9** (1.45 g, 2.3 mmol) was dissolved in 7 mL of CH_2Cl_2, and 7 mL of trifluoroacetic acid (TFA) was slowly added. After 3 hours the reaction was stopped and CH_2Cl_2 and TFA were evaporated under reduced pressure. The product was dissolved in a small amount of CH_2Cl_2 and precipitated from diethyl ether. Any TFA left was removed in a vacuum dessicator over KOH. This yielded 88% (1.17 g of the impure cortisol prodrug **10**. Two spots were observed with TLC using 7.5% of methanol in CH_2Cl_2. The product was purified using silica column chromatography with 15% of methanol in CH_2Cl_2 yielding 494 mg of the pure product (37%). ^{1}H NMR (DMSO) δ = 6.55[38] (s, 1H, NH), 5.92 (s, 1H, NH), 5.56 (s, 1H, C-4), 5.47 (s, 1H, C-17-OH), 5.07 (d, *J*=17.58 Hz, 1H, C-21), 4.74 (d, *J*=16.84 Hz, 1H, C-21), 4.39 (d, *J*=3.67 Hz, 1H, C-11-OH), 4.27 (m, 1H, C-11), 3.42 (d, *J*=3.66 Hz, 2H, C-31), 2.94 (q, *J*=19.04 Hz, 2H, C-27), 2.46 (1H, C-16), 2.41 (1H, C-16), 2.38 (1H, C-2), 2.37 (2H, C-23), 2.18 (1H, C-6), 2.18 (1H, C-2), 2.08 (1H, C-1), 1.92 (1H, C-8), 1.92 (1H, C-7), 1.92 (1H, C-12), 1.77 (1H, C-1), 1.67 (1H, C-15), 1.67 (1H, C-14), 1.67 (1H, C-12), 1.55 (quintet, *J* = 29.29 Hz, 2H, C-24), 1.45 (1H, C-16), 1.37 (s, 1H, C-19), 1.37 (2H, C-26), 1.30 (2H, C-25), 1.30 (1H, C-15), 1.00 (1H, C-7), 0.86 (1H, C-9), 0.76 (s, 3H, C-18). ^{13}C NMR (DMSO) δ = 205.4 (C-20), 198.1 (C-3), 174.4 (C-32), 172.5 (C-22), 172.3 (C-5), 158.1 (C-29), 121.5 (C-4), 88.6 (C-17), 67.4 (C-21), 66.3 (C-11), 55.6 (C-9), 51.6 (C-14), 46.8 (C-13), 44.5 (C-31), 39.0 (C-12), 38.9 (C-10), 34.1 (C-1), 33.5 (C-23), 33.2 (C-16), 33.1 (C-2), 32.8 (C-6), 31.4 (C-7), 31.2 (C-8), 29.8 (C-26), 25.8 (C-25), 24.3 (C-24), 23.3 (C-15), 20.5 (C-19), 16.6(C-18).[37] Elemental analysis; Calculated: C 62.48%, H 7.69%, N 4.86%. Found: C 53.74%, H 6.65%, N 4.02%. This corresponds to 1.5 molecules of CH_2Cl_2 to every cortisol prodrug molecule. Calculated: C 53.74%, H 6.73%, N 3.98%, deviation C 0.00%, H 0.08%, N 0.04%). MALDI-TOF analysis. Calculated: 576. Found (m/z): 599 $[M + Na]^+$, 621 $[M + 2Na]^+$, 656 $[M + Na + NaCl]^+$.

*Ethyl 6-(3-(2-ethoxy-2-oxoethyl)ureido)hexanoate (**11**)*

Ethanol (14 mL) was added slowly to thionylchloride (11.74 mmol) at 0 °C. A suspension containing aminocaproic acid (7.95 mmol) in ethanol (12 mL) was added to this solution and the reaction mixture was stirred for 30 minutes at 0 °C and refluxed for 2-3 hours. The mixture was concentrated under reduced pressure, yielding white foam. The foam was dissolved in DCM and washed three times with 1 M NaOH. The organic layer was dried over Na_2SO_4, filtered and a solution of ethyl isocyanate acetate (8.07 mmol) in DCM (5 mL) was slowly added, the reaction was stirred overnight. The reaction was monitored with IR spectrsoscopy (formation of urea, strong absorption bands were present at 3370 cm^{-1} and 1644 cm^{-1}. Also a strong absorption band was present at 2254 cm^{-1}, indicating the presence of unreacted isocyanate). The solution was concentrated under reduced pressure and the product was precipitated in hexane, after which 1.26 g of a white powder was obtained in 55% yield. The powder was shown to be free of isocyanate by IR spectroscopy. ^{1}H NMR ($CDCl_3$) δ = 5.33 (s, 1H, CNHCO), 5.13 (s, 1H, CNHCO), 4.20 (q, *J*=21.4 Hz, 2H, O$CH_2$$CH_3$), 4.13 (q, *J*=20.9 Hz, 2H, O$CH_2$$CH_3$), 3.97 (d, *J*=3.30 Hz, 2H, NHCH_2CO), 3.70 (t, *J*=11.10 Hz, 2H, CO$CH_2$$CH_2$), 2.29 (t, *J*=14.83 Hz, 2H, CH_2CH_2NH), 1.63 (quintet, *J*=29.67 Hz, 2H, $CH_2CH_2CH_2$), 1.51 (quintet, *J*=29.12 Hz, 2H, $CH_2CH_2CH_2$), 1.35 (m, 2H, $CH_2CH_2CH_2$), 1.27 (m, 6H, 2 x CH_2CH_3). ^{13}C NMR ($CDCl_3$) δ = 173.9 (CO), 171.7 (CO), 158.2 (NHCONH), 61.3 (OCH_2), 60.4 (OCH_2), 42.3 (NCH_2CO), 40.3 (CH_2CH_2N), 34.2 (CO$CH_2$$CH_2$), 29.8 ($CH_2CH_2CH_2$), 26.4 ($CH_2CH_2CH_2$), 24.6 ($CH_2CH_2CH_2$), 14.3 ($CH_2CH_3$), 14.2 ($CH_2CH_3$).

*6-(3-(carboxymethyl)ureido)hexanoic acid (urea-glycine tail **12**)*

The protected urea-glycine tail **11** (66 mg) and 0.2 M NaOH (10 mL, 8 eq) were dissolved in THF (10 mL) and the mixture was stirred for 24 h. THF was removed under reduced pressure and subsequently the pH was lowered to 1.5 using a 1 M HCl solution. Water was removed under reduced pressure. Subsequently acetone was added (4 mL) and the resulting suspension was decanted, this was repeated 6 times, in order to remove NaCl formed by adding HCl. The fractions containing the product (as determined by TLC) were combined and filtered. After evaporation of actone, the crude product was obtained (43 mg). Impurities, like the mono ethyl ester) were removed by trituration; a

mixture of acetone and diethyl ether (1:1) was added and the resulting suspension was decanted, this was repeated three times. The pure product was obtained (35 mg, 66% yield) and analyzed using ^{1}H NMR and ^{13}C NMR spectroscopy and elemental analysis. ^{1}H NMR (400 MHz, D_2O) δ = 3.75 (d, *J*=1.4 Hz, 2H, NH*CH*$_2$COOH), 2.98 (t, *J*=13.2 Hz, 2H, CH_2*CH*$_2$NH), 2.25 (t, *J*=14.7 Hz, 2H, HOOC*CH*$_2$$CH_2$), 1.48 (quintet, *J*=30.0 Hz, 2H, CH_2*CH*$_2$$CH_2$), 1.36 (quintet, *J*=28.6 Hz, 2H, CH_2*CH*$_2$$CH_2$), 1.21 (quintet, *J*=30.0 Hz, 2H, CH_2*CH*$_2$$CH_2$). ^{13}C NMR (D_2O) δ = 179.3 (CH_2COOH), 175.4 (CH_2COOH), 147.1 (NHCONH), 42.0 (NH*CH*$_2$COOH), 40.0 (CH_2*CH*$_2$NH), 34.0 (HOOC*CH*$_2$$CH_2$), 29.0 ($CH_2$*CH*$_2$$CH_2$), 25.7 ($CH_2$*CH*$_2$$CH_2$), 24.2 ($CH_2$*CH*$_2$$CH_2$). Elemental analysis; Calculated: C 46.55%, H 6.94%, N 12.06%. Found: C 45.56%, H 6.60%, N 11.85%.

Glucocorticoid mediated suppression of nitrite production by rat macrophages

In response to cytokines and endotoxins, the inducible nitric oxide synthetase (iNOS) in macrophages is induced, allowing the macrophages to produce a vast amount of NO radicals, which is believed to contribute to the defense against bacteria. Glucocorticoids are known to suppress the induction of iNOS.[28,29] Given its instable nature, nitric oxide will react with components (e.g. oxygen) in the cellular medium. During this process nitrite ions will be produced, that can be measured using the spectrophotometric Griess assay[39,40] as described below.

Macrophages were plated in 24 well plates, essentially as described for the toxicity tests. However additionally, (bacterial) lip polysaccharide (LPS) was added to the cells at a final concentration of 10 µg/ml to induce the i-NOS. Also, cortisol, cortisol-prodrug or cortisol-prodrug-dendrimer complex were added to obtain final concentrations of 0, 1, 3, 10, 30, 100, and 300 nM. Following 72 h of incubation in a humidified atmosphere containing 5% CO_2 at 37 ^{o}C, the amount of nitrite in cell medium was determined using the Griess method as follows: to 0.25 ml of medium, 750 µL of Griess reagent (consisting of 0.5 w/v % sulfanilamine, 0.05 w/v % N-(1-naphthyl)-ethylene-diamine in 14 v/v % acetic acid in milliQ) was added and allowed to react for 10 minutes at room temperature to yield a purple colored azol dye. The amount of nitrite was determined by measuring the absorbance of the reaction product at 540 nm and comparing the value with that of a calibration curve of sodium nitrite in blank cell culture medium (ranging between 0 and 20 µM nitrite). Appropriate blanks were included as well as incubations in which the LPS was omitted to assess basal nitrite production of the macrophages, which for further calculations was subtracted from the nitrite production in the presence of LPS. Effects of test substances were calculated from the ratios of nitrite produced by iNOS induction in the presence or absence of test-substance. No effect of the dendrimers on basal or LPS induced nitrite formation was observed in the macrophages.

2.6. REFERENCES

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CHAPTER 3

SYNTHESIS OF MULTIVALENT PEPTIDE DENDRIMERS*

ABSTRACT

A general synthetic method is developed for the conjugation of synthetic thioester-peptides to cysteine-functionalized dendrimers using native chemical ligation. The first, second and third generation cysteine dendrimers have successfully been prepared and several thioester-peptides are synthesized by solid phase peptide synthesis. Ligation of peptides to the first generation is successful for all peptides and products can be isolated in good yields. Second generation peptide dendrimers can be obtained and isolated as well, whereas the synthesis of third generation peptide dendrimers presents several problems in both the analysis and isolation of the products. The modular approach presented here provides access to a wide variety of well-defined multivalent peptides that are attractive for understanding the fundamental mechanisms of multivalency in biological interactions and for potential use in biomedical applications in targeted drug delivery, molecular imaging and immunology.

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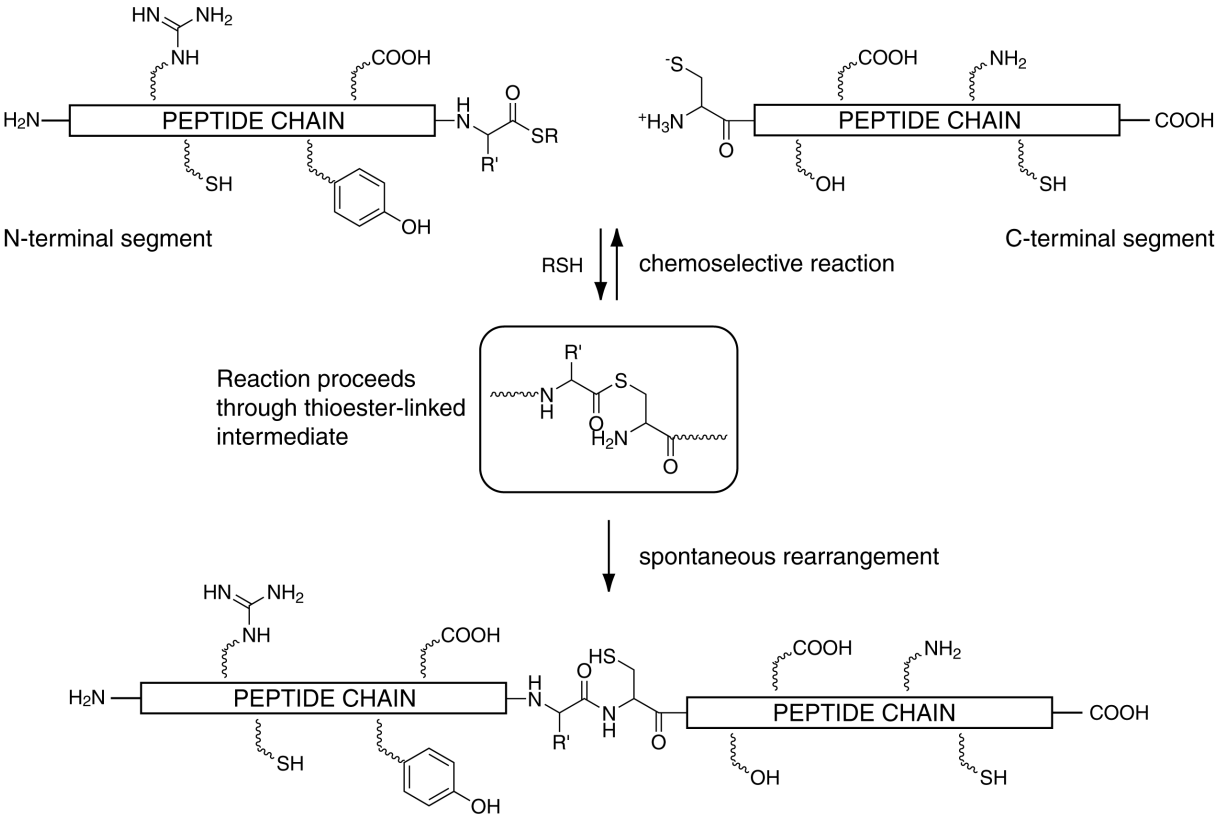
3.1. NATIVE CHEMICAL LIGATION AS A SYNTHETIC TOOL

There are several strategies for preparing peptide dendrimers (Chapter 1): conventional peptide synthesis carried out in solution,[1-4] solid phase methods[5-11] introduced by Merrifield, or a hybrid solid-phase/solution approach.[12,13] Reversed phase high performance liquid chromatography (RP-HPLC) is routinely used to purify these synthetic products and electrospray ionization mass spectrometry (ESI-MS) has provided a straightforward general method for the precise characterization of the covalent structure of unprotected synthetic peptides.[14,15] Thus, synthetic peptide chemistry, whether by stepwise SPPS or by solution methods, can provide routine access to polypeptide chains of about 50 amino acid residues in length.

Peptide ligation methods provide a practical and powerful means to assemble synthetic peptides into proteins. The peptides can be protected or unprotected, and coupling can occur in an aqueous or organic solvent, in solution or on a solid support. This is based on the ability to routinely make unprotected peptides of less than 50 amino acids in length, and consists of a practical way to join synthetic peptides to yield polypeptides of any desired length, and, subsequently, the corresponding folded proteins.

Due to the unprotected nature of the peptides, ligation reactions can be performed in aqueous solution to which guanidine can be added to increase the solubility of the reacting peptide segments, thereby allowing the use of higher peptide concentrations to accelerate the ligation reaction. Chemical ligation methods have proven to be simple to implement, highly effective and generally applicable. A variety of ligation chemistries have been used and the chemical ligation of unprotected peptide segments has provided access to a wide range of protein targets. Although in most cases an unnatural structure at the site of ligation between two peptide segments was formed (thioester-forming ligation,[16] oxime-forming ligation,[17] thioether-forming ligation,[18] directed disulfide formation,[19,20] thiazolidine-forming ligation[16,21-27]), these unnatural structures are often well tolerated within a folded protein, and numerous examples exist of active protein molecules that are chemically synthesized in these ways.[28-31]

Currently, the most common ligation method is native chemical ligation (Scheme 3.1). Wieland *et al.* developed the chemical foundation for this reaction in 1953,[32-34] when the reaction of ValSPh and CysOH in aqueous buffer was shown to yield the dipeptide ValCysOH. In 1994, Dawson and Kent *et al.* introduced an ingenious extension of this reaction to ligate large peptide fragments.[32,35] In this method, simply mixing two peptide segments that contain designed, mutually reactive functionalities led to the formation of a single polypeptide product containing a native peptide bond at the ligation site. This highly chemoselective reaction is performed in aqueous solution at neutral pH under denaturing conditions.



Scheme 3.1 *Native Chemical Ligation. Unprotected peptide segments are reacted by means of reversible thiol/thioester exchange to give a thioester-linked intermediate. This intermediate is able to undergo a spontaneous nucleophilic rearrangement by a highly favored intramolecular mechanism; this step is irreversible under the conditions used, and gives a polypeptide product that is linked by a native peptide bond. The product can be folded to give the desired synthetic protein molecule.*

The thioester intermediate undergoes a spontaneous rearrangement via intramolecular nucleophilic attack to give the desired amide-linked product.[32,36] The result is a completely native polypeptide chain that is obtained directly in its final form. The ligation is independent of the amount of cysteine residues in the chain[37] and no protective groups are necessary for any of the side-chain functional groups normally found in proteins. In addition, no racemization can be detected at the site of ligation,[38-42] and quantitative yields of the ligation product are obtained.

Native chemical ligation has allowed for the chemical synthesis of moderately-sized proteins by multistep ligation of several peptide fragments, the synthesis of proteins with synthetic moieties such as fluorescent dyes, biotin tags, and the immobilization of peptides and proteins on material surfaces.[43,44] The application of native chemical ligation was recently made available to recombinantly expressed proteins by the development of expression systems based on self-cleavable intein domains that generate proteins containing *N*-terminal cysteine or *C*-terminal thioester groups.[45-48]

As mentioned before, native chemical ligation is the chemoselective reaction of two unprotected segments that quantitatively yields the ligated product with a native peptide bond at the site of ligation. In the next section (3.2), the synthesis of cysteine dendrimers and thioester-peptides as starting materials necessary for ligation will be described. Subsequently, coupling of peptides to first (section 3.3), and higher generation dendrimers (section 3.4) are reported to evaluate whether the application of native chemical ligation is suitable as an attractive and general synthetic strategy to conjugate oligopeptides to dendrimers.

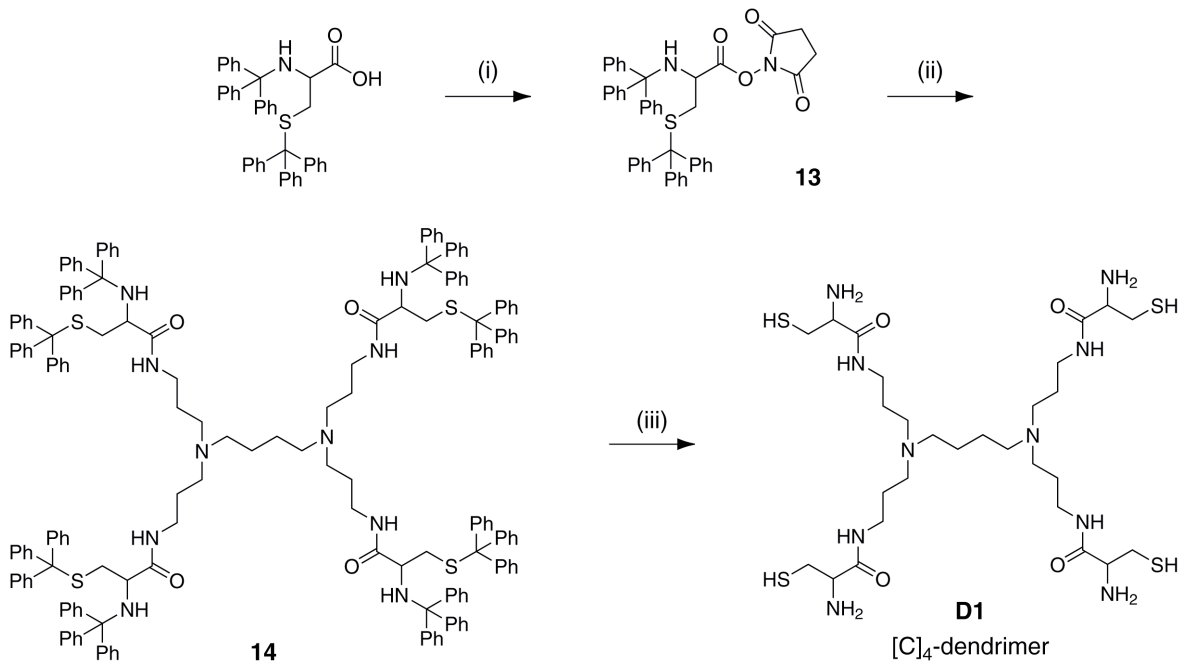
3.2. SYNTHESIS OF STARTING MATERIALS

To allow native chemical ligation to poly(propylene imine) dendrimers, two strategies are possible: dendrimers with thioester-residues at the periphery ligated with *N*-terminal cysteine-peptides, or dendrimers with cysteine residues at the periphery ligated with C-terminal thioester-peptides. The latter strategy is chosen because the synthesis of cysteine dendrimers is more straightforward and thioester–dendrimers are highly instable compounds.[49]

3.2.1 SYNTHESIS OF CYSTEINE DENDRIMERS

N-terminal cysteine dendrimers were one of the two components necessary for native chemical ligation and were synthesized according to the following procedure. For the synthesis of the first, second and third generation of cysteine dendrimers, the carboxylic acid of cysteine (protected with a trityl group at the thiol and amine) was activated (Scheme 3.2). This was done by activation with HBTU and subsequent reaction with hydroxysuccinimide to yield the succinimide ester of cysteine (**13**). The product **13** was purified by washing with acidic water and obtained in 89% yield. This activated and protected cysteine residue **13** was then coupled to the amine end groups of the first, second and third generation poly(propylene imine) dendrimer in dichloromethane in the presence of base (resulting in compound **14** in case of first generation dendrimer). Finally, the trityl protecting groups were removed by reaction with TFA. Washing with diethyl ether and subsequent lyophilization of the water layer resulted in the crude product.

The crude product still contained some TFA, as was measured using ^{19}F NMR. Peaks were observed corresponding to the TFA-salt (presumably with the amines of the dendrimer) and of free TFA. To remove these TFA contaminations, the cysteine dendrimers were further purified using RP-HPLC. After lyophilization, the first generation cysteine dendrimer **D1** was obtained in 76% yield, the second generation cysteine dendrimer **D2** was obtained in 60% yield and the third generation cysteine dendrimer **D3** in 28% yield (Figure 3.1).



Scheme 3.2 *Synthesis of first generation of cysteine-functionalized poly(propylene imine) dendrimers. Reaction conditions: (i) O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), N-hydroxysuccinimide, Et_3N, DMF, (ii) first generation poly(propylene imine) dendrimer, Et_3N, dichloromethane, (iii) TFA, triethylsilane (2.5 v%), water (2.5 v%).*

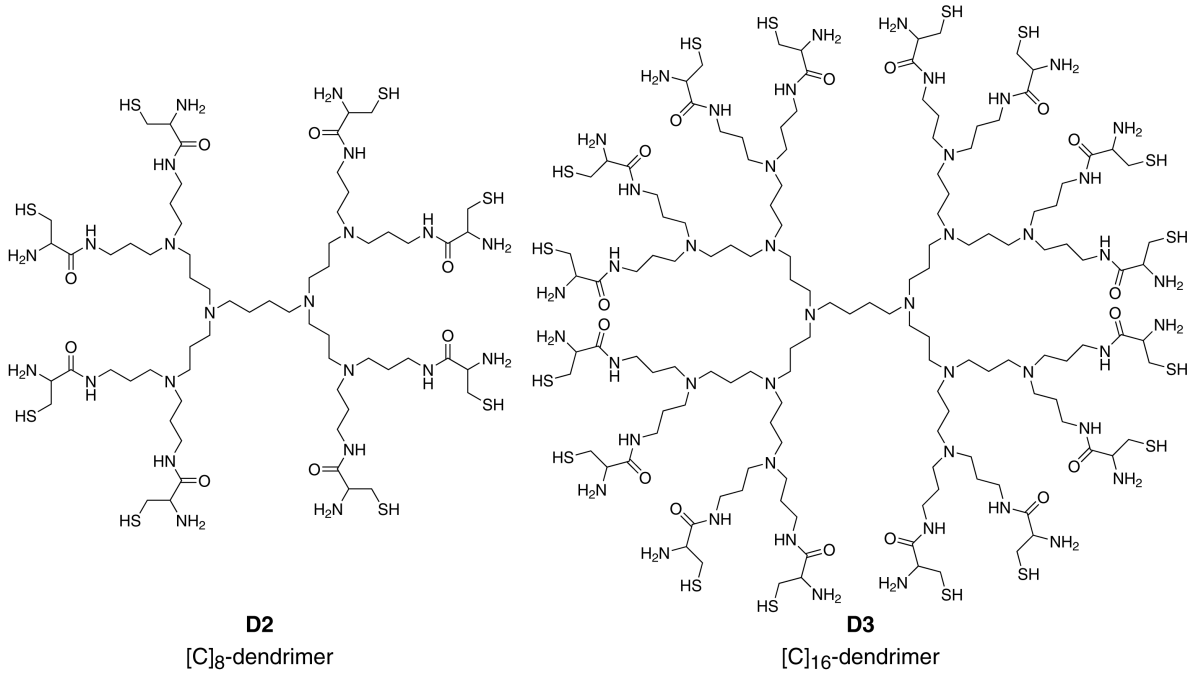


Figure 3.1. *Structural formula of second (**D2**) and third generation (**D3**) of cysteine-functionalized poly(propylene imine) dendrimers.*

Deprotection of the trityl-protected amine and thiol groups using TFA and scavengers at room temperature yielded the cysteine-functionalized dendrimers **D1**, **D2**, and **D3**, but also some byproducts as observed by ESI-MS (in Figure 3.2 the mass spectrum of **D2** is shown).

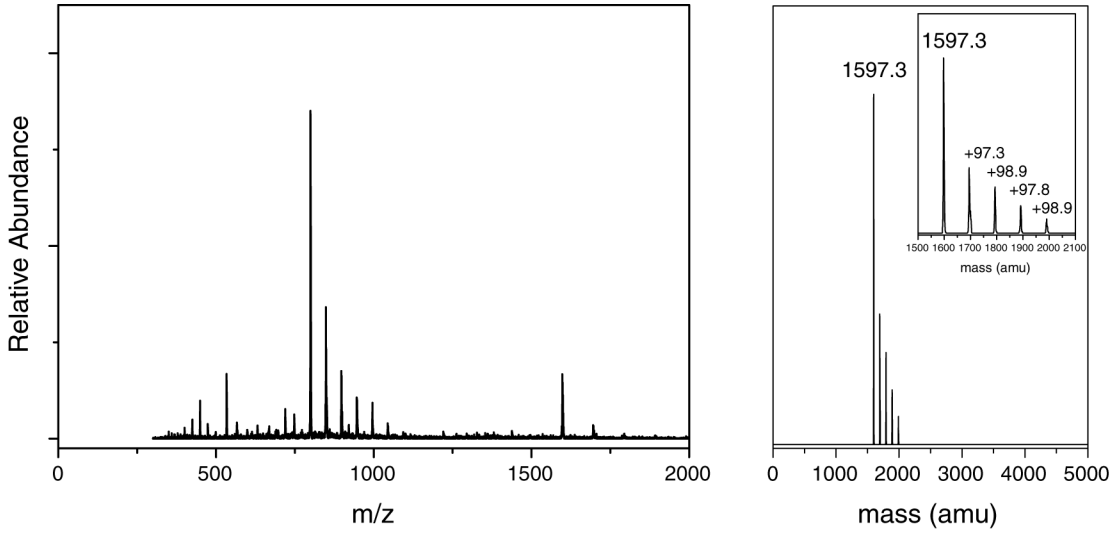
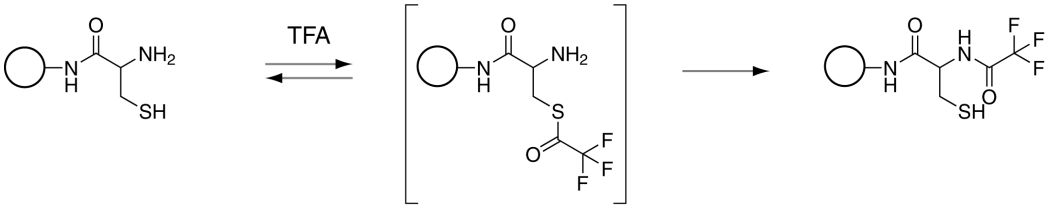


Figure 3.2 *ESI-MS spectrum and corresponding deconvoluted spectrum of [C]$_8$-dendrimer **D2**, when removal of trityl groups was done at room temperature; byproducts are seen with incremental mass of ca. 97 Da.*

For instance, in the ESI-MS spectrum of **D2** byproducts with incremental mass of ca. 97 Da were seen. This could correspond to the addition of a trifluoroacetyl group to the amine of the cysteine residues. A suggested mechanism for the formation of these byproducts is given in Scheme 3.3.



Scheme 3.3 *Suggested mechanism for the formation of trifluoroacetyl groups at the periphery of the dendrimers during deprotection at ambient temperature.*

After the trityl groups are removed by TFA, the free thiol of cysteine is able to react with TFA that is present in excess in the reaction mixture. A thioester intermediate is formed that spontaneously rearranges by intramolecular attack of the free amine of cysteine at the carbonyl of the trifluoroacetyl residue, to form a trifluoroacetylated amine.

For the higher generations of dendrimer, more reactive groups are present at the periphery. As a result, the amount of byproducts will increase exponentially with the number of end groups. For example, a molecule with only one reactive site shows a conversion of 96% into the desired product, for a first generation dendrimer (having four end groups), the conversion is $0.96^4 = 85\%$. For the second generation (eight end groups) and third generation (sixteen end groups) the conversion becomes $0.96^8 = 72\%$ and $0.96^{16} = 52\%$, respectively. This

calculation shows that the higher the generation of dendrimer becomes, the more important it is to have high-yielding reactions. This was confirmed in the ESI-MS spectra of the products **D1**, **D2** and **D3**, when the trityl groups were deprotected at room temperature (Figure 3.2).

Performing the deprotection with TFA and some scavengers at low temperature could solve the problem of trifluoroacetyl adducts. At low temperatures, TFA is still capable of removing the trityl protective groups, but the amount of trifluoroacetyl adducts is expected to decrease. Instead of performing the deprotection at room temperature, it was carried out at 0 °C. Also, during work-up the temperature was kept at 0 °C. ESI-MS of **D1**, **D2** and **D3** showed that no trifluoroacetyl groups added to the dendrimers were obtained (Figure 3.4). The spectra were measured in the presence of β-mercaptoethanol. Under these circumstances, only the thiols of **D1** and **D2** were fully reduced. In the case of **D3**, a mass was found that corresponds to **D3** with one disulfide bridge. For a detailed study on the oxidation and reduction behavior of the sulfur bonds in the dendrimers, the reader is referred to Chapter 4 of this thesis.

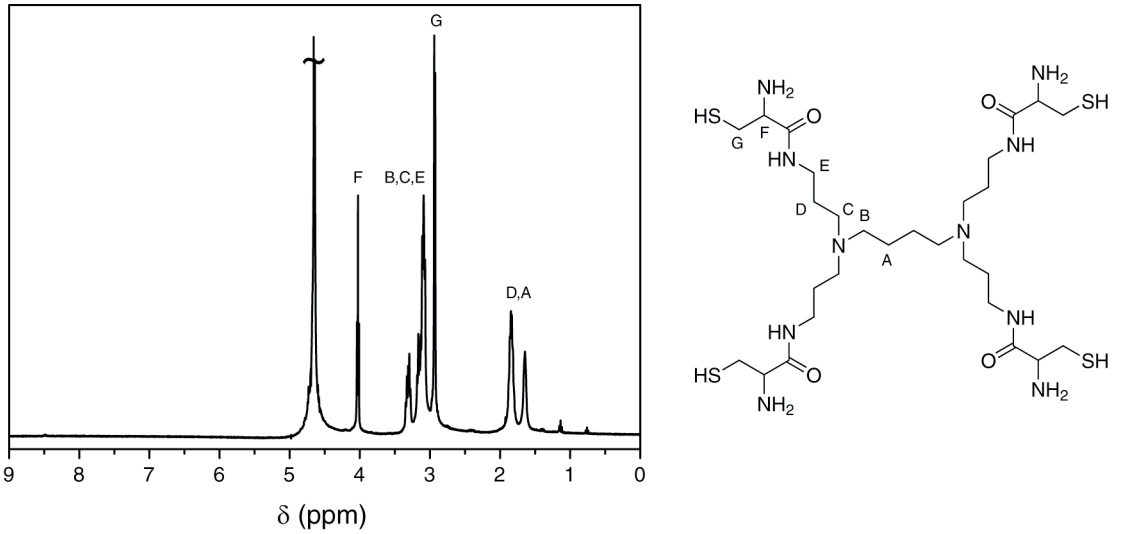


Figure 3.3 *^{1}H-NMR [C]$_4$-dendrimer in D_2O. The peak at 4.7 ppm is from water.*

The cysteine dendrimer **D1** was also characterized by ^{1}H-NMR spectroscopy (Figure 3.3). As frequently seen with NMR spectra of dendrimers, the peaks were somewhat broad. Protons from the interior of the dendrimer and in the middle of a "dendrimer arm" were shown between 2 and 1.5 ppm (A and D). The doublet at 2.9 ppm corresponded to the protons of CH_2 groups of cysteine (G) and the triplet at 4.0 ppm corresponded to the CH of cysteine (F). Furthermore, protons of all CH_2 groups next to an amine were shown between 3.5 and 3 ppm (B, C and E). Clearly, no signals were present from trityl groups between 7-8 ppm, indicating the deprotection with TFA was successful.

First, second and third generation cysteine dendrimers **D1-D3** were successfully synthesized in good yields. The side reaction of trifluoroacetyl addition during the removal of the trityl groups could be suppressed by lowering the reaction temperature.

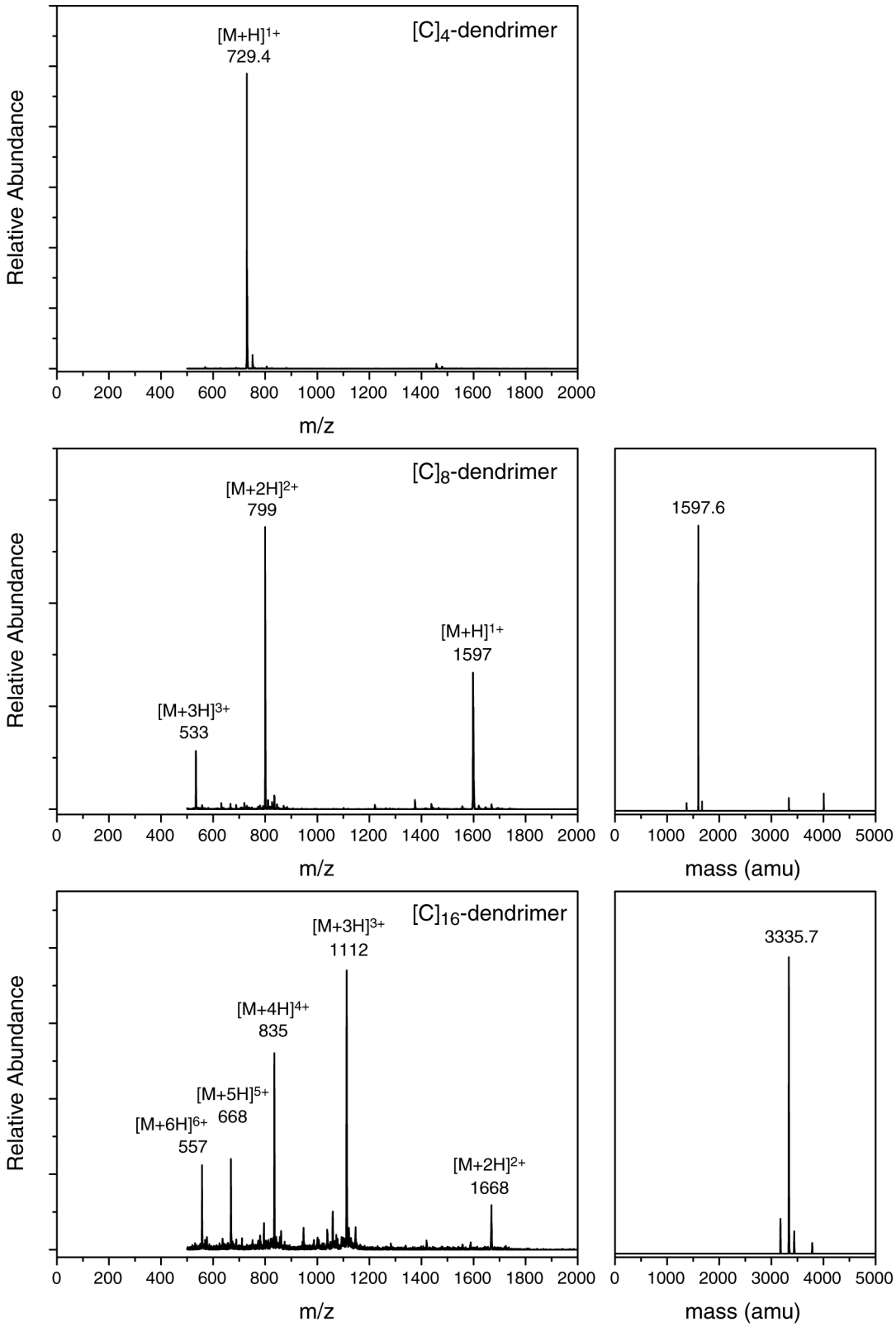
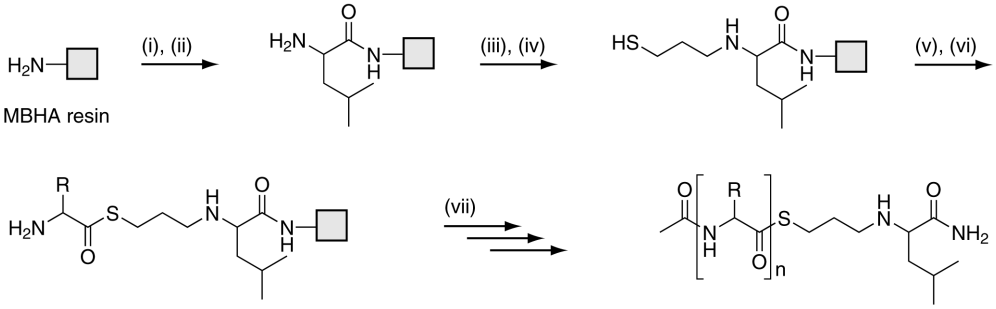


Figure 3.4 *ESI-MS spectra and corresponding deconvoluted spectra of* ***D1*** *(calculated mass: 729.1 Da),* ***D2*** *(calculated mass: 1598.4 Da), and* ***D3*** *(calculated mass: 3337.1 Da) under reducing conditions.*

3.2.2 SYNTHESIS OF THIOESTER-PEPTIDES

Chemical synthesis of *C*-terminal thioester-peptides for use in native chemical ligation on *N*-terminal cysteine dendrimers was achieved using *tert*-butyloxycarbonyl (*t*Boc) amino acid derivatives employing HBTU activation *in situ* neutralization protocols for *t*Boc-mediated solid phase peptide synthesis (SPPS) on a 4-methylbenzhydryl amine (MBHA) resin (Scheme 3.4).[42] Starting from the MBHA resin, leucine is coupled for 30 minutes following standard procedures. Subsequently, MPA is activated with HBTU; this is coupled for 30 minutes to the resin in DMF with DIPEA. The trityl-group is removed using a mixture of TFA with some scavengers (a solution of 2.5% triisopropylsilane and 2.5% water in TFA is used). The thioester is obtained upon addition of the next amino acid to the 3-mercaptopropionylamide-leucine-MBHA resin using standard procedures. All amino acids are coupled for 10 minutes, only arginine and asparagine residues are coupled for 20 minutes. A repetitive addition and deprotection of the desired amino acids and cleavage with anhydrous hydrogen fluoride yields the *C*-terminal thioester peptide with an amide at the *C*-terminus. Purification was done using preparative RP-HPLC and the product was analyzed by ESI-MS. In this thesis, the thioester peptides will be abbreviated, using the one-letter code of the corresponding amino acids, and MPA for mercaptopropionic acid, *e.g.* AcGG-MPAL: *N*-Acetyl-glycine-glycine-(mercaptopropionic acid)-leucine (see appendices).



Scheme 3.4 *Solid Phase Peptide Synthesis (SPPS) of C-terminal thioester peptides. Reaction conditions: (i)* tert-*butyloxycarbonyl leucine, HBTU, diisopropylethylamine (DIPEA), dimethylformamide (DMF); (ii) trifluoroacetic acid (TFA); (iii) 3-(tritylmercapto)propionic acid (MPA), HBTU, DIPEA, DMF; (iv) TFA, 2.5% triisopropylsilane, 2.5% water; (v) any* tert-*butyloxycarbonyl-protected amino acid, HBTU, DIPEA, DMF; (vi) TFA; (vii) repetitive addition of amino acids followed by cleavage from the resin with hydrogen fluoride. Purification of the resulting peptide was done using RP-HPLC.*

Several thioester peptides were synthesized following this synthetic methodology (Figure 3.5). Some of them served as model peptides, like AcGG-MPAL **P1** and AcLYRAG-MPAL **P2**. Different functional groups were present in the side chains of **P2** to obtain a good model peptide.[42] In addition, for the biological application of these peptides, some thioester-peptides with possible biological activity were synthesized. The peptide AcGRGDSGG-MPAL **P3** was synthesized, as the RGD sequence is known to bind integrins at the cell surface and is frequently used in targeting studies.[50] In combination with PHSRN,[51] RGD

showed even stronger binding to integrins. Therefore the thioester–peptide AcPHSRNGG-MPAL **P4** was also synthesized. The peptide sequence KLVFF is part of the β-amyloid protein found in patients with Alzheimer's disease and is known to form β-sheets.[52] The peptide is used in aggregation and toxicity studies of the β-amyloid protein. For that reason, the thioester-peptide AcKLVFFGG-MPAL **P5** was synthesized. Studies concerning the biological application of these peptides are discussed in Chapter 5 of this thesis. The peptides were purified using RP-HPLC and analyzed with ^{1}H-NMR spectroscopy, ^{1}H,^{1}H-COSY NMR spectroscopy and ESI-MS.

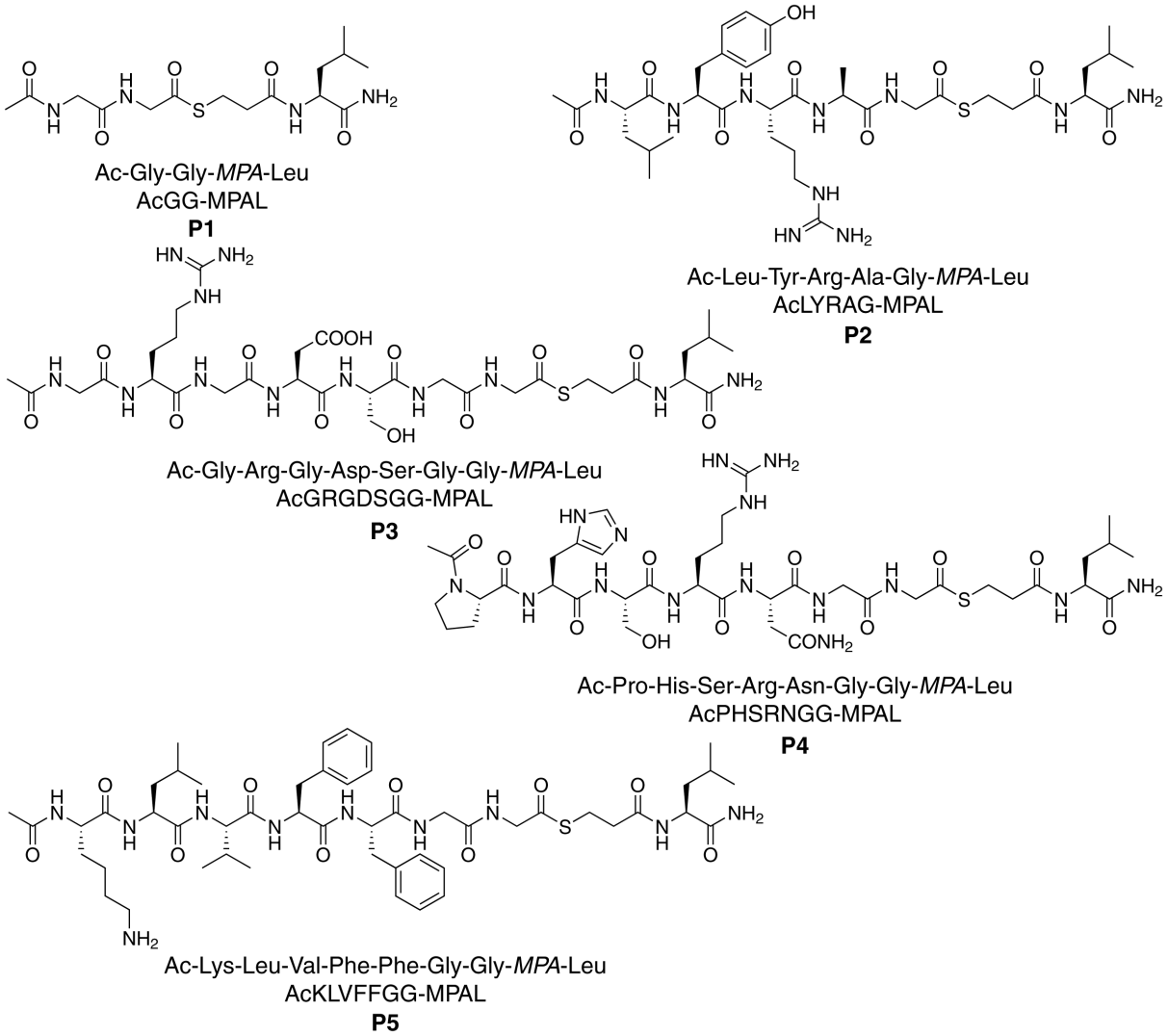


Figure 3.5 *Structural formula of C-terminal thioester-peptides synthesized and used in this research.*

The analysis of **P2** is described in more detail, the synthesis of **P1**, **P3**, **P4** and **P5** was similar to **P2** and can be found in the experimental section. After HF cleavage of the peptide from the resin and purification using RP-HPLC, the pure thioester-peptide **P2** was obtained in 78% yield as a white fluffy powder. **P1-P4** appeared to be water-soluble peptides, whereas **P5** showed low solubility in water. When the product was analyzed with ESI-MS (Figure 3.6) a peak was found at 821.4 Da ($[M+H]^+$ that corresponded to the calculated mass (821.0 Da) of

the thioester-peptide **P2**. All masses of the thioester-peptides **P1**-**P5** shown in Figure 3.5 corresponded well (Table 3.1) to their calculated masses. In the synthesis of **P4** histidine was used that had a 3,5-dinitrophenyl (DNP) protective group at the side chain. As this group was not removed during HF cleavage, the final thioester-product still had a DNP group attached to the histidine. However, under ligation conditions, the DNP group was removed due to the reducing environment.

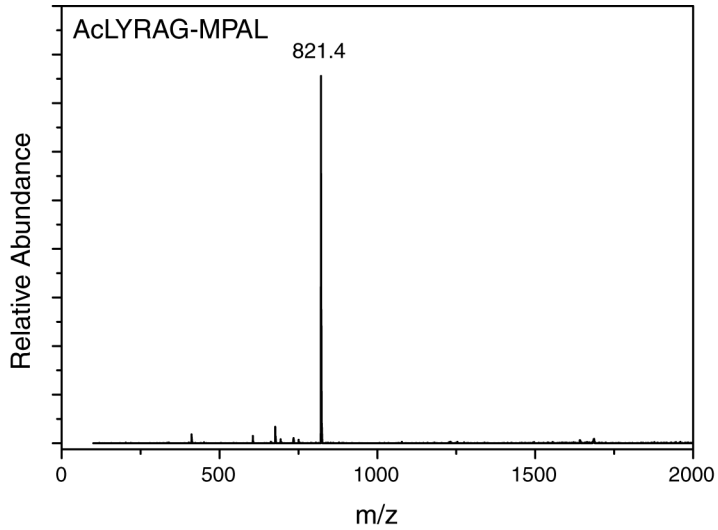


Figure 3.6 *ESI–MS spectrum (m/z) of AcLYRAG-MPAL **P2**.*

Table 3.1 *Calculated and observed masses of thioester-peptides **P1**-**P5**.*

Thioester-peptide		*Obs. mass (Da)*[a]	*Calc. mass (Da)*	*Yield (%)*
AcGG-MPAL	**P1**	375.0	374.4	53% (80 mg)
AcLYRAG-MPAL	**P2**	821.4	821.0	78% (93 mg)
AcGRGDSGG-MPAL	**P3**	847.5	846.9	84% (92 mg)
AcPHSRNGG-MPAL	**P4**	1132.4[b]	1132.1	56% (75 mg)
AcKLVFFGG-MPAL	**P5**	1009.6	1009.2	81% (128 mg)

[a] $[M+H]^+$ masses are shown. [b] The mass of the peptide with a 3,5-dinitrophenyl (DNP) protective group at the histidine residue is shown.

Apart from ESI-MS, the thioester-peptides were also characterized by ^{1}H-NMR spectroscopy, gradient ^{1}H,^{1}H-COSY NMR spectroscopy and ^{13}C-NMR spectroscopy. It was difficult to assign all the peaks exactly, due to overlap of the peaks (experimental section). In the ^{1}H-NMR spectrum of AcLYRAG-MPAL (Figure 3.7) the signals of tyrosine (Y) were shown separately; two doublets around 7 ppm from the phenyl group, a triplet of the CH proton at 4.6 ppm and two multiplets around 3 ppm that corresponded to the CH_2 protons. In addition, two triplets for MPA protons were observed at 2.6 ppm and 3.2 ppm. Underneath the last mentioned peak, the signal from one of the three CH_2 protons of arginine was also present. Between 4.5 ppm and 4 ppm the CH proton of arginine, leucine (twice) and alanine and the CH_2 protons of glycine overlapped.

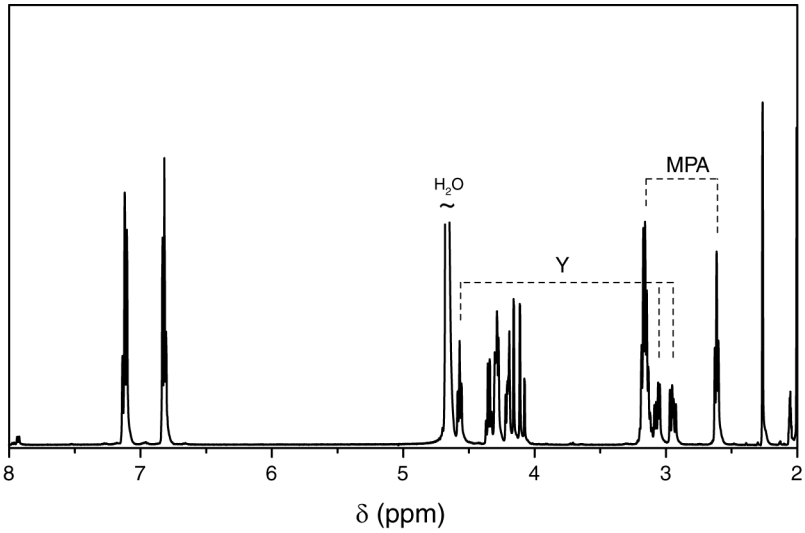


Figure 3.7 *Part of the ^{1}H-NMR spectrum of AcLYRAG-MPAL **P2** in D_2O.*

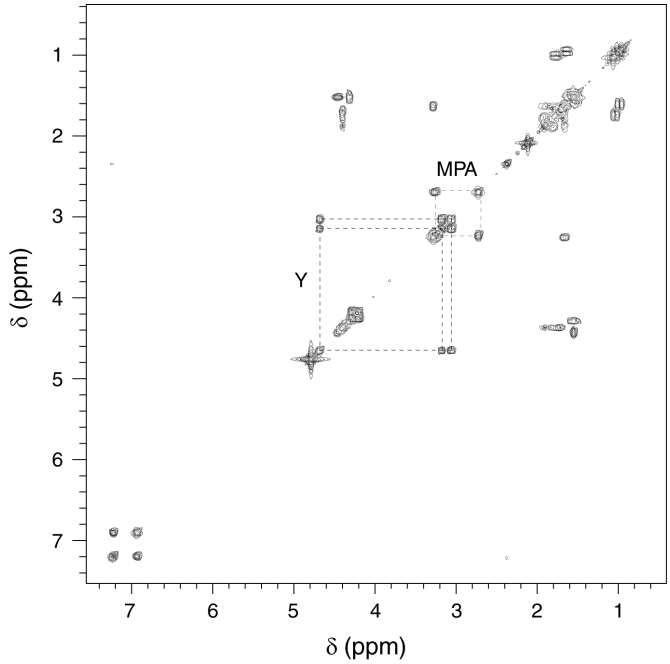


Figure 3.8 *^{1}H,^{1}H-COSY NMR of AcLYRAG-MPAL **P2**. Crosspeaks are highlighted of the protons of the CH_2 and CH groups of tyrosine (Y). Also the crosspeak of the two CH_2 groups of MPA is highlighted.*

Generally, cross peaks can be seen from protons attached to carbons that are next to each other in a gradient ^{1}H,^{1}H-COSY NMR spectrum. Figure 3.8 showed the gradient ^{1}H,^{1}H-COSY NMR of AcLYRAG-MPAL. For reasons of clarity, only a few of the cross peaks were highlighted; the cross peak of CH of tyrosine coupled to both of the multiplets of CH_2 of tyrosine. Furthermore, a cross peak was seen between the two peaks corresponding to CH_2 of mercaptopropionic acid. In the region between 2 ppm and 1 ppm peaks were seen corresponding to several CH_2 groups in the side chains of the peptide (from arginine and leucine) that resulted in cross peaks to neighboring CH groups at 4.2-4.6 ppm.

In short, thioester-peptides **P1–P5** were synthesized in good yields using *t*Boc-mediated SPPS. The peptides were analyzed using RP-HPLC, ESI-MS, ^{1}H-NMR spectroscopy and ^{1}H,^{1}H-COSY NMR spectroscopy.

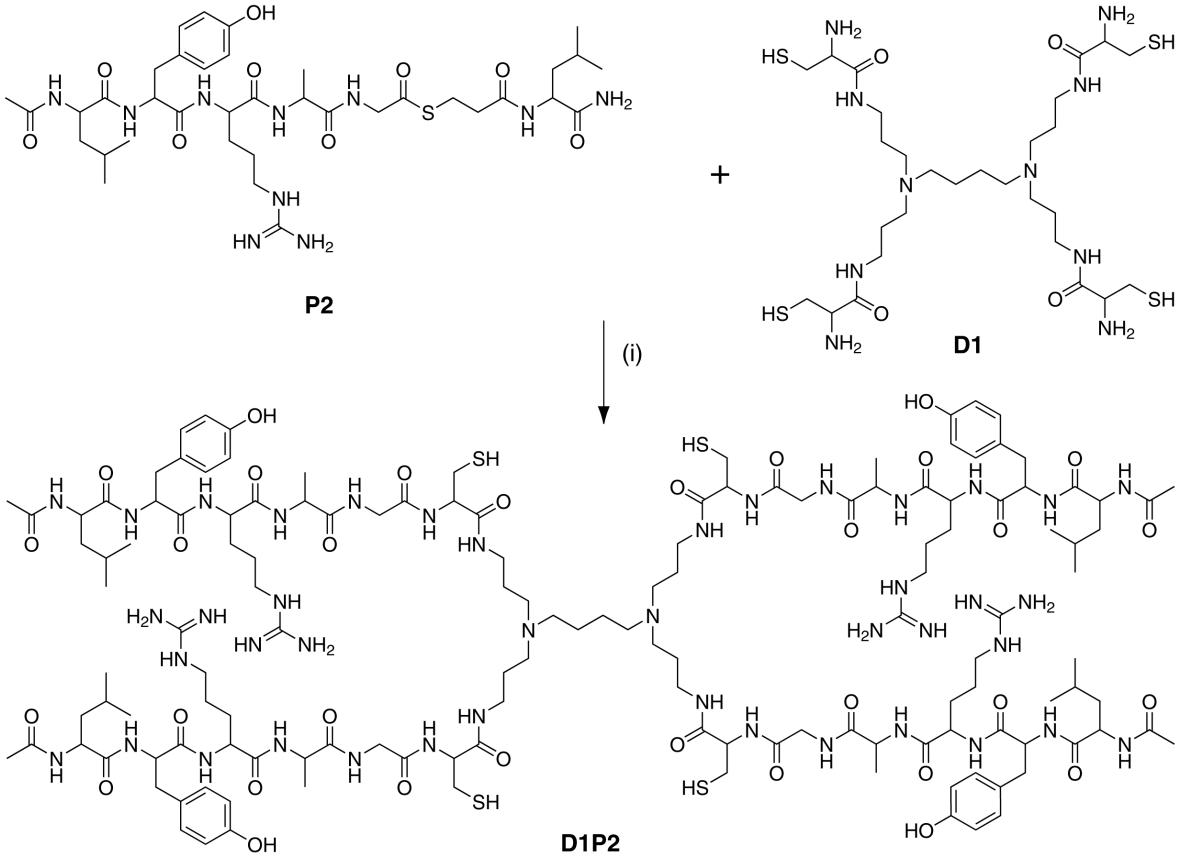
3.3. LIGATION TO FIRST GENERATION CYSTEINE DENDRIMERS

The coupling of several oligopeptides to the first generation cysteine dendrimer **D1** *via* native chemical ligation is described. As the ligation of non-water-soluble thioester-peptides to dendrimers comprises a different synthetic strategy from those which are water-soluble, they are described in different sections (3.3.1 and 3.3.2). In addition, different peptides are coupled to one dendrimer (section 3.3.3), as an example for purposes where synergistic binding of peptides is favored. Finally, thioester-peptides having an additional functionality (biohybrids) are synthesized to assess ligation efficiency (section 3.3.4).

3.3.1 LIGATION OF WATER-SOLUBLE OLIGO-PEPTIDES

A first generation cysteine dendrimer **D1** was combined with several water-soluble thioester-peptides. Ligation of two peptide fragments is typically performed in an aqueous buffer containing guanidine at neutral pH, thiophenol and benzylmercaptan are added to accelerate the rate of reaction. In this case, **D1** was dissolved in a tris(hydroxymethyl)aminomethane hydrochloride (Tris) buffer at pH 8.0 containing 6 M guanidine and added to 4–5 equivalents of **P2**. After adding thiophenol and benzylmercaptan, and adjusting the pH to 7–7.5 if necessary, the reaction was carried out at 37 °C for 1 hour. This resulted in the formation of the peptide-dendrimer $[AcLYRAGC]_4$-dendrimer **D1P2** (Scheme 3.5). RP-HPLC combined with ESI–MS analysis was performed to analyze the reaction. The final product was purified using RP-HPLC.

The HPLC-chromatogram showed the presence of product **D1P2** as well as **P2**, and if the peptide was used in excess, the benzylmercaptan exchanged peptide product. Furthermore, full conversion of dendrimer **D1** was observed as a sharp peak of the product in the HPLC chromatogram. When **D1** was added in excess, the intermediate products ($[AcLYRAGC]_3$-dendrimer, $[AcLYRAGC]_2$-dendrimer, $[AcLYRAGC]_1$-dendrimer) were also seen. The *m/z* spectrum of the HPLC trace corresponding to **D1P2** is shown in Figure 3.9. The spectrum showed the desired molecule **D1P2** with $[M+6H]^{6+}$ = 524 Da; $[M+5H]^{5+}$ = 628 Da; $[M+4H]^{4+}$ = 786 Da; $[M+3H]^{3+}$ = 1047 Da. After deconvolution of this spectrum, a mass of 3139.7 Da was obtained which corresponded to the calculated mass of 3139.8 Da, indicating that the correct product was formed (Figure 3.9).



Scheme 3.5 *Native chemical ligation of four equivalents of* ***P2*** *with one equivalent of* ***D1****. For clarity reasons, the stereochemistry of the peptides is not shown; all amino acids are in the L-form. Reaction conditions: (i) 2% thiophenol, 2% benzylmercaptan, 0.1 M Tris buffer, 6 M guanidine (pH 7.0-7.5), 1 hour, 37 °C.*

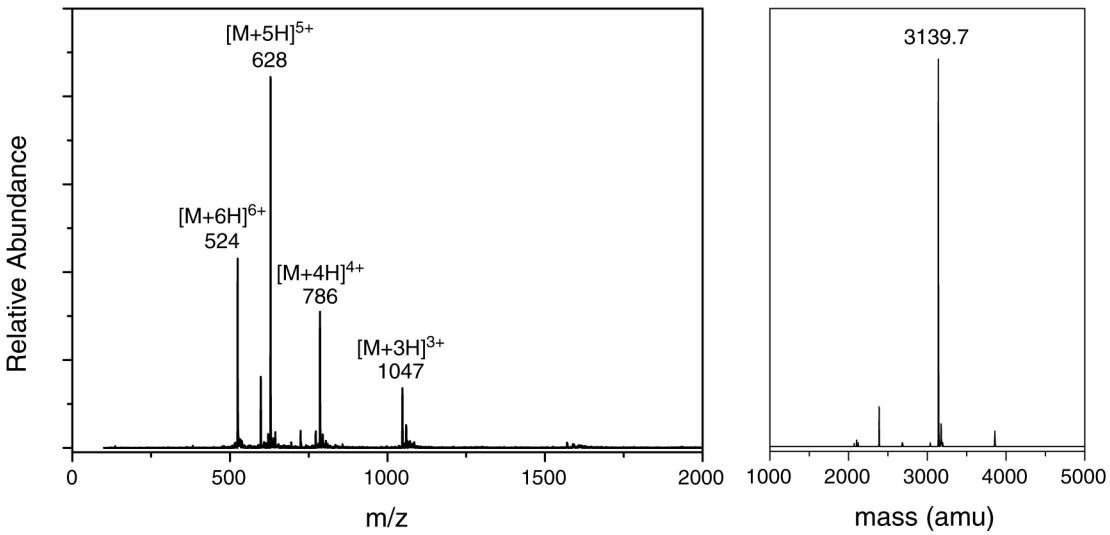


Figure 3.9 *ESI-MS spectrum and corresponding deconvoluted spectrum of* ***D1P2*** *(calculated mass: 3139.8 Da).*

The other thioester-peptides that were synthesized: AcGG-MPAL **P1**, AcGRGDSGG-MPAL **P3** and AcPHSRNGG-MPAL **P4**, showed the same behavior as **P2** during the ligation reaction. After one hour, the ligation was complete, showing only the tetramer products $[AcGGC]_4$-dendrimer **D1P1**, $[AcGRGDSGGC]_4$-dendrimer **D1P3** and $[AcPHSRNGGC]_4$-dendrimer **D1P4**. The products were analyzed with ESI-MS and purified using RP-HPLC. The *m/z* spectrum of **D1P1** trace showed the desired product with a deconvoluted mass of 1353.0 Da, and corresponded well to the calculated mass (1353.6 Da). The *m/z* spectrum of **D1P3** trace showed the desired product with a deconvoluted mass of 3243.2 Da that corresponded well to the calculated mass (3243.5 Da). In the *m/z* spectrum of **D1P4** trace, peaks were seen of the product with a deconvoluted mass of 3720.4 Da that corresponded well to the calculated mass (3720.1 Da). In all cases, full conversion of the starting material **D1** was observed and a sharp peak corresponding to the product was seen in the chromatogram. The peptide dendrimers **D1P1** and **D1P3** were purified using RP-HPLC and obtained in 36-53% and 67% yield, respectively.

Table 3.2 *Calculated and observed masses of several peptide-dendrimers.*

Peptide-dendrimer		*Obs. mass (Da)*	*Calc. mass (Da)*	*Yield (%)*
$[AcGGC]_4$-dendrimer	**D1P1**	1353.0	1353.6	67% (12 mg)
$[AcLYRAGC]_4$-dendrimer	**D1P2**	3139.7	3139.8	*n.i.**
$[AcGRGDSGGC]_4$-dendrimer	**D1P3**	3243.2	3243.5	36-53% (27 mg)
$[AcPHSRNGGC]_4$-dendrimer	**D1P4**	3720.4	3720.1	*n.i.**

*not isolated

The fact that several water-soluble thioester-peptides with different functional side chains could be attached to dendrimers via native chemical ligation opens the door to a range of multivalent peptide dendrimers. These dendrimers could be isolated in good yields. Following sections illustrate the scope and limitations of this technique.

3.3.2 LIGATION OF NON-WATER-SOLUBLE OLIGO-PEPTIDES

So far, the thioester-peptides **P1-P4** used in native chemical ligation reactions with dendrimers were all water-soluble. The thioester-peptide AcKLVFFGG-MPAL **P5** is highly insoluble in aqueous buffers: even in 6 M guanidine the peptide was hardly soluble. As a result, native chemical ligation of thioester **P5** with **D1** showed different behavior compared to the water-soluble thioester-peptides discussed in the previous section.

The dendrimer **D1** was dissolved in Tris buffer containing 6 M guanidine and the resulting solution was added to an excess of peptide **P5**, the mixture was vigorously stirred for 5 minutes, after which a precipitate remained, and later identified as thioester-peptide **P5**. Thiols were added and the mixture was vigorously stirred and heated to 37 °C. After one hour, the mixture was analyzed using RP-HPLC. Despite the low solubility of the peptide, the product $[AcKLVFFGGC]_4$-dendrimer **D1P5** was formed as indicated by the ESI-MS

spectrum of the corresponding HPLC trace (Figure 3.10). After deconvolution, the mass (3892.3 Da) was in good agreement with the calculated mass (3892.9 Da).

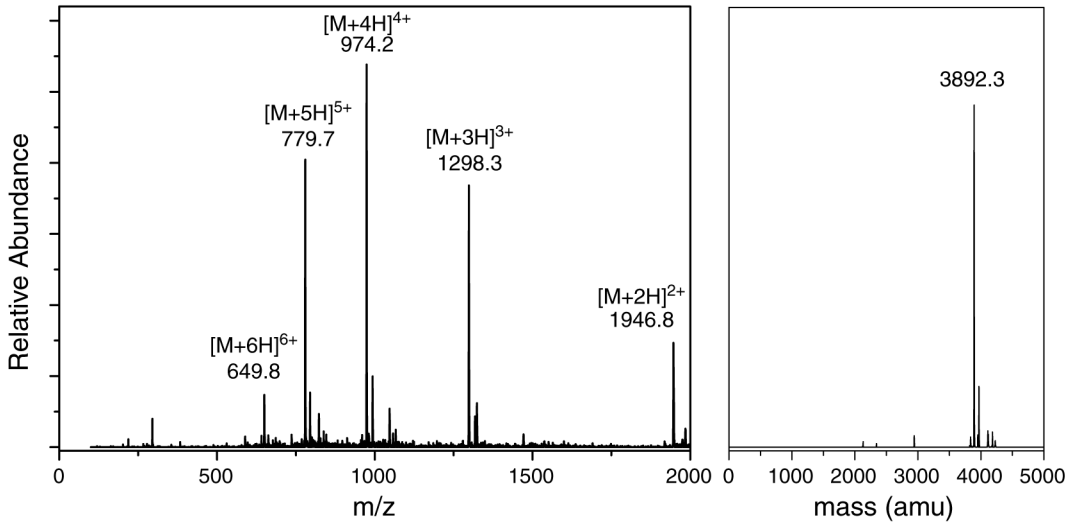


Figure 3.10 *ESI-MS spectrum and corresponding deconvoluted spectrum of* ***D1P5*** *(calculated mass: 3892.9 Da).*

However, the ligation reaction did not go to completion as concluded from the HPLC chromatogram (Figure 3.11a); the intermediate products $[AcKLVFFGGC]_3$-dendrimer, $[AcKLVFFGGC]_2$-dendrimer and $[AcKLVFFGGC]_1$-dendrimer were identified by ESI-MS. Even after several days or after adding more thioester-peptide, the reaction did not proceed to completion, probably due to the low solubility of the thioester-peptide and the peptide dendrimer products.

Table 3.3 *Calculated and observed masses of* $[AcKLVFFGGC]_n$*-dendrimers.*

Peptide-dendrimer		*Obs. mass (Da)*	*Calc. mass (Da)*
$[AcKLVFFGGC]_4$-dendrimer	**D1P5**	3892.3	3892.9
$[AcKLVFFGGC]_3$-dendrimer		3101.3	3101.9
$[AcKLVFFGGC]_2$-dendrimer		2310.9	2311.0
$[AcKLVFFGGC]_1$-dendrimer		1519.6	1520.0

In an effort to improve the solubility of the peptide, four ligation reactions were performed on analytical scale in different solvent mixtures. In all four reactions, the dendrimer **D1** was dissolved in Tris buffer containing 6 M guanidine and added to an excess of the thioester-peptide **P5**. Then, the mixture was vigorously stirred, in the first reaction, no cosolvent was added, in the second reaction 10% (v/v) of DMSO was added, in the third reaction, 10% (v/v) of acetonitrile was added and in the fourth reaction, 10% (v/v) of DMF was added. The reactions with organic cosolvent were all clear solutions, whereas the reaction without organic solvent contained precipitated material. Thiophenol and benzylmercaptan were added and the ligations were carried out at 37 ^{o}C for one hour. Analysis was performed using RP-HPLC and ESI-MS.

In all cases the initial thioester-peptide and exchanges thereof (indicated with a P, Figure 3.11) are shown in the total ion current (TIC) trace, this is because the thioester-peptide was added in excess to the reaction mixture. The ligation with no added organic solvent shows all the intermediate products, and the reaction did not go to completion. However, for the ligations in which 10% of an organic solvent was added, the reaction did go to completion, as no intermediate products were seen in any of these reactions. When acetonitrile was used, the ESI-MS spectrum showed the product peak, but also the desired product with several molecules of acetonitrile attached. The results for the ligation in DMF and DMSO were similar, however it was preferred to use DMF as the cosolvent, as removal of DMF from the reaction mixture is more straightforward that the removal of DMSO.

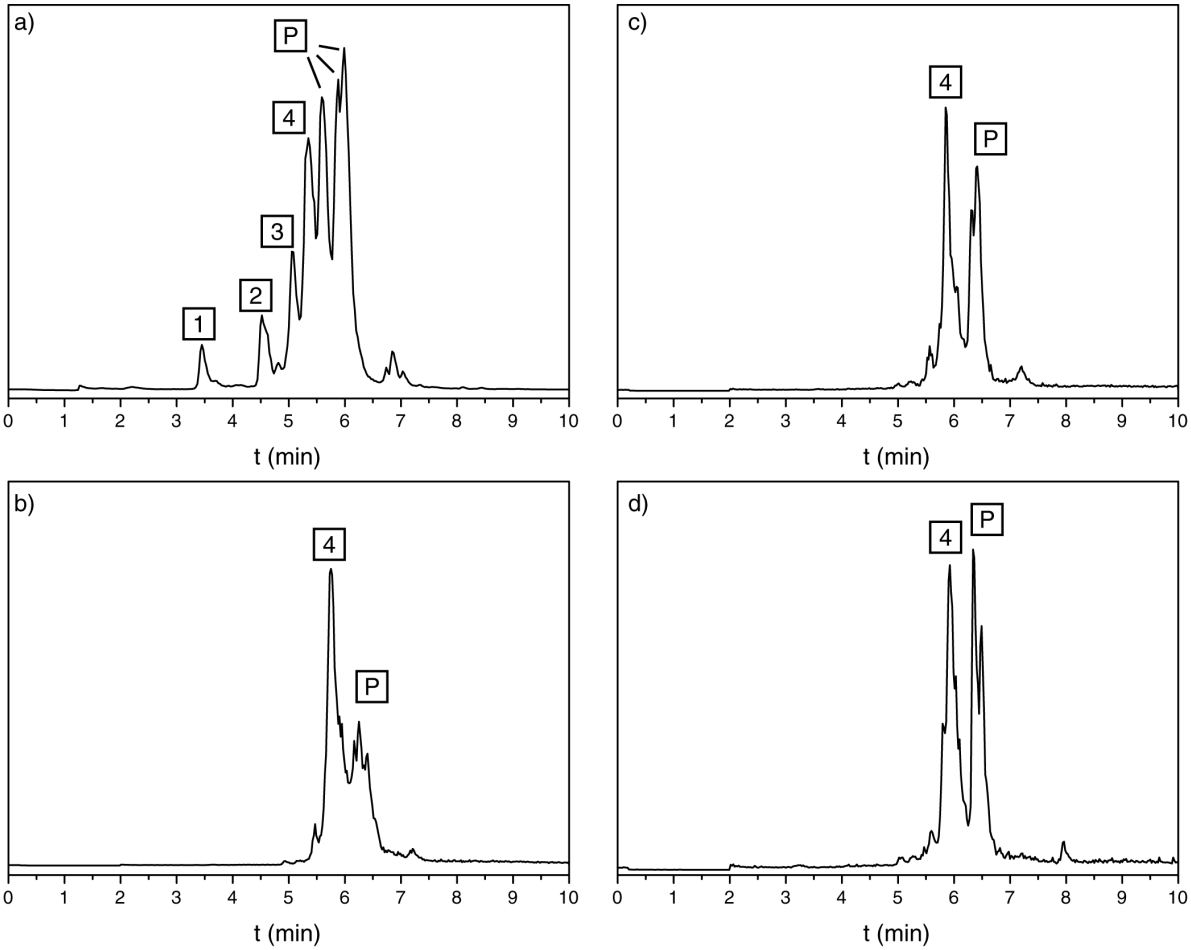


Figure 3.11 *TIC traces of ligation of AcKLVFFGG-MPAL* ***P5*** *with [C]$_4$-dendrimer* ***D1*** *using different reaction media: a) 6 M guanidine HCl; b) 6 M guanidine HCl, 10% dimethylsulfoxide; c) 6 M guanidine HCl, 10% dimethylformamide; d) 6 M guanidine HCl, 10% acetonitrile. P = thioester-peptide and thioester exchanges thereof, numbers correspond to the number of peptides attached to the dendrimer scaffold (4 means full conversion).*

By performing the ligation reaction of **P5** to **D1** on preparative scale using standard ligation conditions and 10% DMF, the dendrimer **D1P5** could not be isolated. When measuring on

analytical RP-HPLC column, the product peak was identified with ESI-MS, however, using a preparative RP-HPLC column, no such peak was observed.

To increase the solubility of the thioester peptide **P5**, the corresponding thioester-peptide without *N*-terminal acetyl group KLVFFGG-MPAL **P5'** was synthesized using *t*Boc-mediated SPPS, purified using RP-HPLC and isolated in 58% yield. The resulting thioester-peptide **P5'** showed good water-solubility and ligation to the first generation cysteine dendrimer **D1** could be performed using standard ligation conditions (Tris buffer pH 7.5, 6 M guanidine, 2 v% thiophenol, 2 v% benzylmercaptan, 37 °C) and the dendrimer **D1P5'** was obtained in 25% yield (Table 3.4). In Chapter 5, the effect of this peptide-dendrimer on aggregation behavior and toxicity of the β-amyloid peptide is reported.

Table 3.4 *Observed and calculated mass of peptide and corresponding peptide-dendrimer.*

Peptide(-dendrimer)		Obs. mass (Da)	Calc. mass (Da)	Yield (%)
KLVFFGG-MPAL	**P5'**	967.5	967.2	58% (50 mg)
$[KLVFFGGC]_4$-dendrimer	**D1P5'**	3725.2	3724.8	25% (12 mg)

By altering the ligation conditions, a thioester-peptide with low water-solubility could successfully be ligated to the first generation cysteine dendrimer. Preparative scale purification of the resulting peptide dendrimer has not yet been successful on RP-HPLC and the optimal conditions still must be found. By removal of the *N*-terminal acetyl group of the peptide, the solubility in water was enhanced and preparative purification of the peptide dendrimer product using RP-HPLC was achieved.

3.3.3 LIGATION OF SEVERAL OLIGO-PEPTIDES TO ONE DENDRIMER

For specific applications, it could be beneficial to have different peptides attached to one single scaffold. For example, it was known that PHSRN is a synergistic peptide-sequence to RGD.[51,53] In other words, by placing PHSRN in proximity to RGD, the binding of RGD to integrins in the cell membrane became stronger. Therefore, a molecule was synthesized with both oligopeptides attached to the same scaffold. As a first attempt, two equivalents of AcGRGDSGG-MPAL **P3** and two equivalents of AcPHSRNGG-MPAL **P4** were added to one equivalent of **D1** on an analytical scale.

The reaction was performed on an analytical scale (0.49 mg, 1.0 eq **D1**, 1.2 mg, 2.1 eq **P3**, 1.6 mg, 2.1 eq **P4**) under standard ligation conditions (10 mg/mL peptide, 2% thiophenol, 2% benzylmercaptan, Tris buffer at pH 7.0 containing 6 M guanidine, 37 °C, 1 h). On HPLC, total conversion of the dendrimer was observed. The product was further analyzed with ESI-MS, and the *m/z* spectrum contained a lot of peaks that were found to correspond to **D1P3** after deconvolution (Figure 3.12). However, after deconvolution, peaks could be related to different peptide-dendrimer constructs for which the following abbreviation is used: $\mathbf{D1(P3)_n(P4)_m}$ where n and m are the number of peptides attached. The products $\mathbf{D1(P3)_4}$ (=**D1P3**), $\mathbf{D1(P3)_3(P4)_1}$, $\mathbf{D1(P3)_2(P4)_2}$ and $\mathbf{D1(P3)_1(P4)_3}$ were observed with masses of 3244.2

Da, 3362.8 Da, 3481.8 Da and 3602.1 Da, respectively (calculated masses: 3243.5 Da, 3362.6 Da, 3481.8 Da and 3601.0 Da, respectively). Surprisingly, no **D1(P4)$_4$** could be found. The differences in observed product yields were difficult to explain since the peptides used were equal in length and solubility and had the same residues at the site of ligation (GG-MPAL). However the remaining residues were different and could be a reason for the difference in observed reactivity.

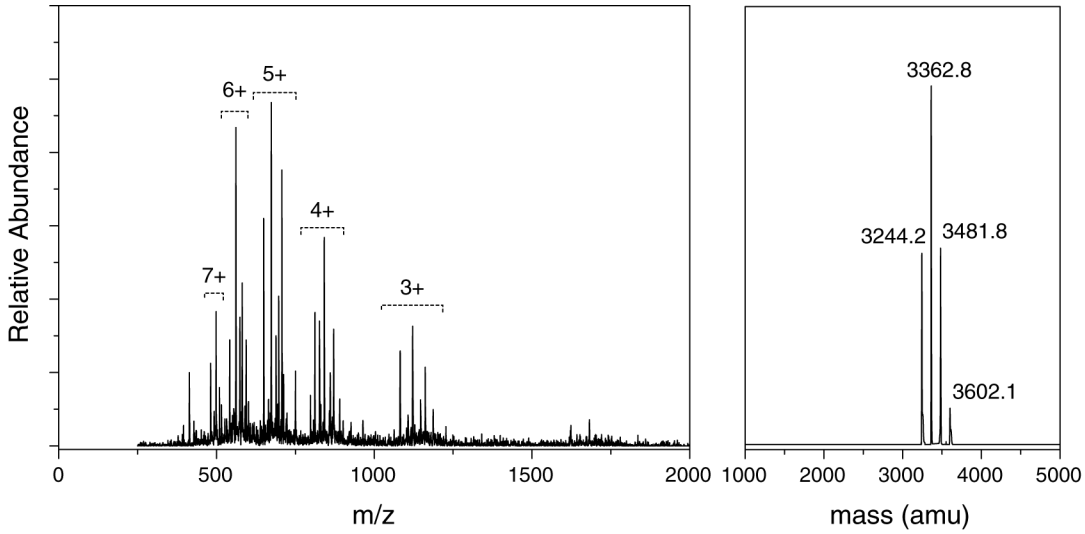


Figure 3.12 *ESI-MS spectrum and corresponding deconvoluted spectrum of peptide dendrimers with different peptides attached.*

Peptide dendrimers with different attached peptides were successfully synthesized on analytical scale and as expected, a mixture of products was obtained. A difference in reactivity for the different peptides was observed. In some cases it will be of importance to have a perfectly well-defined peptide dendrimer consisting of an exact amount of different peptides. In Chapter 4, a strategy for the synthesis of such dendrimers is described.

3.3.4 LIGATION OF BIOHYBRIDS

Apart from biologically active peptides, native chemical ligation for peptides with a non-biological chemical functionality (biohybrids) is assessed. This concept is tested using two model systems. First, the "click-system", using the urea-glycine tail as described in Chapter 2 of this thesis is applied (Figure 3.13).[54] To this end, a peptide with a *C*-terminal thioester and an *N*-terminal urea-glycine (**P6**) is synthesized (section 3.3.4.1). The peptide is attached to the dendrimer via the *C*-terminus. It is expected that the *N*-terminus interacts with the interior of the peptide-dendrimer. This can be a driving force for the peptide to adopt a β-hairpin conformation necessary for immune response *in vivo*. These *in vivo* studies are described in Chapter 5.

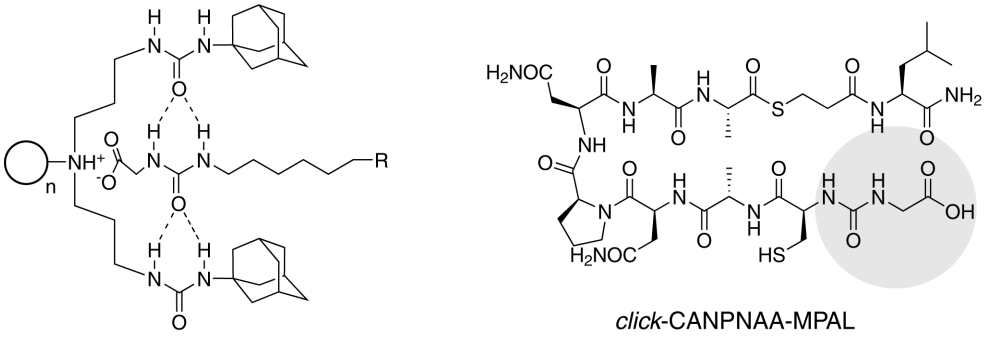


Figure 3.13 *Left: Electrostatic and hydrogen bonding interactions of a click-tail with the periphery of an adamantyl-dendrimer. Right: structural formula of* ***P6*** *having a urea-glycine part for interaction with the interior of the dendrimer (shown in gray circle).*

Second, a 2-ureido-4-pyrimidinone group (UPy group) will be introduced through native chemical ligation (section 3.3.4.2).[55] Supramolecular polymers based on this quadruple hydrogen bonding moieties are known to produce bioactive materials.[56] Particularly, the reversible nature of the hydrogen bonds allows for a modular approach for gaining control over cellular behavior and activity both *in vitro* and *in vivo*. It is expected that dendrimers with one UPy and three RGD sequences can be used for multivalent attachment of cells to the supramolecular UPy polymer surfaces.

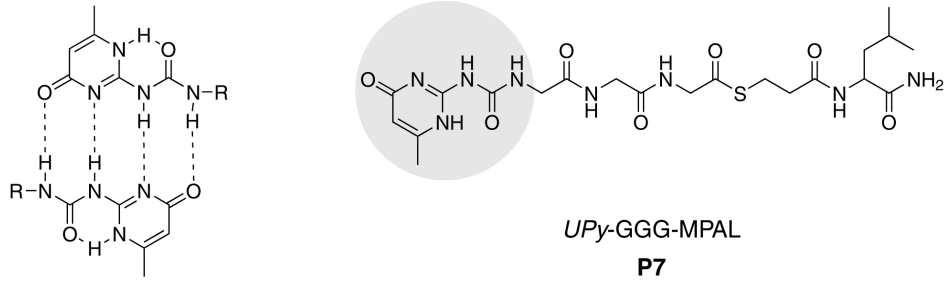
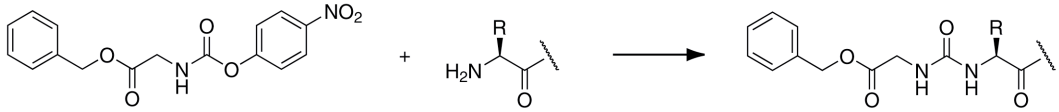


Figure 3.14 *Left: Hydrogen bonding interactions of self-complementary quadruple hydrogen bonding units. Right: structural formula of* ***P7*** *having the quadruple hydrogen-bonding unit 2-ureido-4-pyrimidinone (UPy, shown in gray circle).*

3.3.4.1 THE "CLICK–SYSTEM"

In a first study, the possibility to combine native chemical ligation with a urea-glycine ("click") tail was investigated. The product was purified and studied for biological use (Chapter 5). The thioester-peptide **P6** was synthesized using *t*Boc-mediated SPPS similar to previously described thioester-peptides. In the last step of the synthesis of **P6**, an activated carbamate **15** was added that reacts with the *N*-terminal amine of the cysteine by release of *p*-nitrophenol (Scheme 3.6). When cleaving the peptide from the resin using HF, the benzyl ester was removed and the free acid form of **P6** was obtained. The product was analyzed using ESI-MS.



Scheme 3.6 *Attachment of a urea-glycine tail to CANPNAA-MPAL peptide sequence via carbamate* ***15*** *(kindly provided by B. F. M. de Waal).*

The thioester-peptide **P6** was introduced to the dendrimer scaffold using standard ligation conditions (Tris buffer containing 6 M guanidine in the presence of thiophenol and benzylmercaptan). The product was analyzed using RP-HPLC combined with ESI-MS. The *m/z* spectrum of the product trace showed peaks at $[M+3H]^{3+}$ = 1233 Da and $[M+2H]^{2+}$ = 1849 Da. After deconvolution this resulted in a peak with a mass of 3694.8 Da, whereas the calculated mass of [clickCANPNAAC]$_4$-dendrimer **D1P6** is 3700.1 Da. The difference in found and calculated mass likely originates from disulfide bonds in the product. The peak in the *m/z* spectrum at 1481 Da indicates the presence of a product dimer (found mass: 7391.3 Da; calculated mass 7400.2 Da).

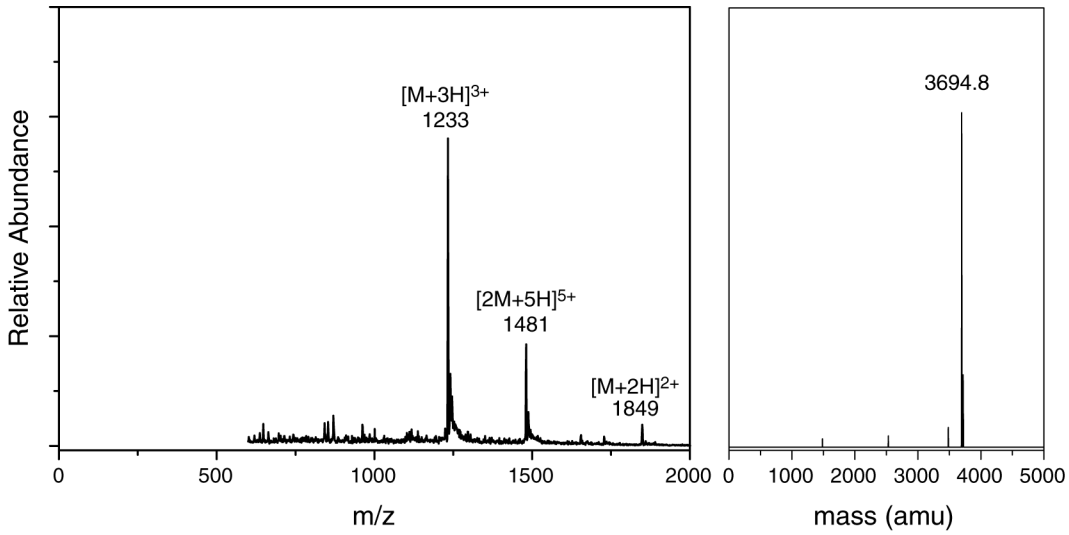


Figure 3.15 *ESI-MS spectrum and corresponding deconvoluted spectrum of [clickCANPNAAC]$_4$-dendrimer.*

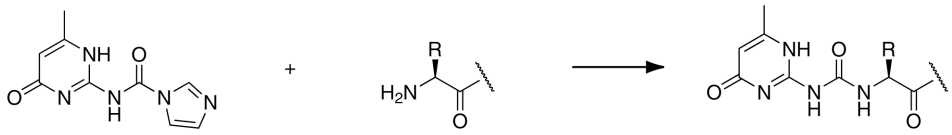
Table 3.5 *Observed and calculated mass for* ***P6*** *and* ***D1P6****.*

Thioester-peptide		*Obs. mass (Da)*	*Calc. mass (Da)*	*Yield (%)*
*click*CANPNAA-MPAL	**P6**	961.3	961.0	36% (48 mg)
[*click*CANPNAAC]$_4$-dendrimer	**D1P6**	3694.8	3700.1	20% (9 mg)

The synthesis of a peptide dendrimer with a biohybrid ("click") system was successfully prepared on a preparative scale. The resulting dendrimer **D1P6** was tested *in vivo* as a synthetic vaccine against malaria (Chapter 5).

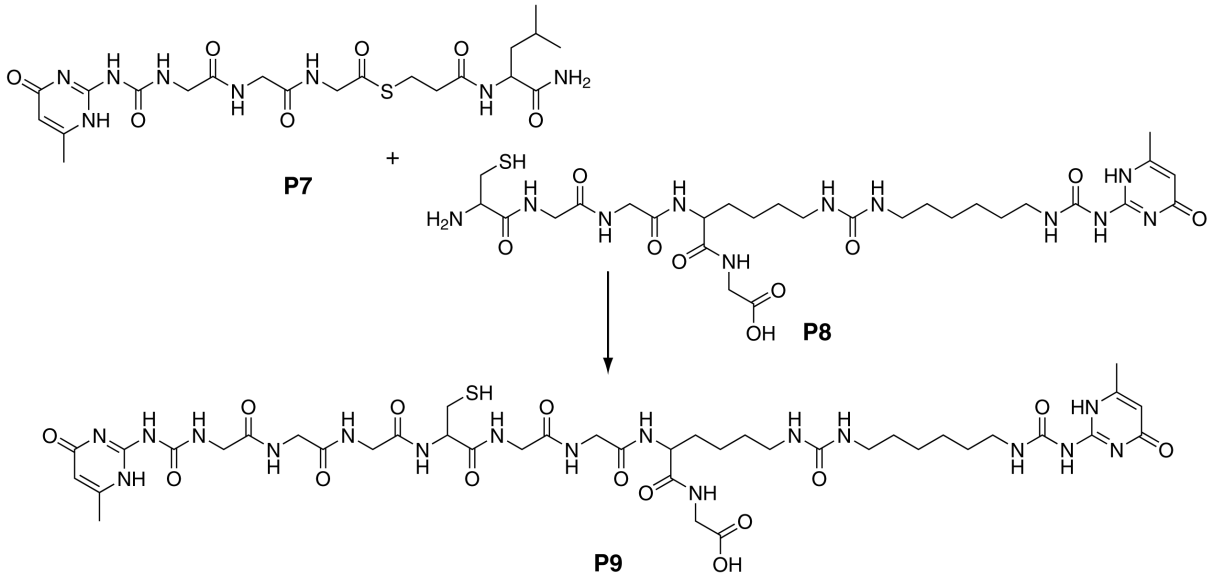
3.3.4.2 A QUADRUPLE HYDROGEN BONDING UNIT

In this section a study to evaluate the possibility of combining native chemical ligation with one or more UPy groups is described. The study aims to investigate whether synthetic access can be established; no products were purified or studied for their biological effects. The UPy group was attached to the *N*-terminus of the peptide sequence GGG-MPAL by reaction with a CDI-activated isocytosine **16**[57,58] (Scheme 3.7), yielding **P7**. Upon displacement of imidazole by an amine, the UPy unit is obtained. The thioester-peptide **P7** was purified with RP-HPLC and analyzed using ESI-MS. The calculated and found masses corresponded well as shown in Table 3.6, indicating the desired product was indeed formed.



Scheme 3.7 *Attachment of the UPy group to the GGG-MPAL peptide sequence.*

To study if the UPy group was inert under the ligation conditions a model system was first prepared (Scheme 3.8, **P9**). The ligation reaction of **P7** to **P8** was performed using standard ligation procedures (10 mg/mL, 2% thiophenol, 2% benzylmercaptan, Tris buffer containing 6 M guanidine, pH 7, 37°C, 1 h) and the reaction was monitored using RP-HPLC.



Scheme 3.8 *Ligation of UPy-GGG-MPAL **P7** with CGGK(UPy)G **P8** (kindly provided by P.Y.W. Dankers).*

The *m/z* spectrum of the trace for **P9** showed peaks corresponding to the mass of the product with one or two protons attached. After deconvolution, the found mass (1035.3 Da) corresponded to the calculated mass (1036.0 Da) of *UPy*-GGGCGGK(*UPy*)G. Apparently, the UPy group is stable under native chemical ligation conditions. Therefore, the synthesis of **D1P7** followed the same procedure. The multivalent *UPy*-dendrimer **D1P7** was made by ligation of **P7** to **D1** under standard ligation conditions with 10% of DMF for solubility

purposes (see section 3.3.2). After one hour the ligation mixture was analyzed using RP-HPLC combined with ESI-MS. The *m/z* spectrum of the trace corresponding to the dendrimer showed peaks corresponding to the product with two or three protons attached. After deconvolution, the found mass (2017.3 Da) corresponded to the calculated mass (2018.2 Da), indicating the desired product was formed.

Furthermore, it could be useful to have one UPy group attached to a dendrimer scaffold and the rest of the end groups of the dendrimer functionalized with peptide groups. This molecule could then be mixed with a supramolecular biomaterial based on UPy groups to have a multivalent binding site for cell adhesion. This dendrimer was synthesized starting from AcGRGDSGG-MPAL **P3** and and performing a ligation reaction with a small excess of $[C]_4$-dendrimer. A statistical mixture was obtained consisting of the dendrimer with two, three, and four peptides attached. This mixture was purified using preparative RP-HPLC and $[AcGRGDSGGC]_3[C]_1$-dendrimer was isolated. Subsequently, native chemical ligation was performed with this dendrimer and UPyGGG-MPAL **P7**, resulting in $[AcGRGDSGGC]_3[UPyGGGC]_1$-dendrimer **D1P3P7**. This product was analyzed with ESI-MS and after deconvolution the found mass (2936.2 Da) corresponded to the calculated mass (2937.1 Da), indicating the desired product was formed.

Table 3.6 *Ligation products of UPyGGG-MPAL.*

Ligation product		Obs. mass (Da)	Calc. mass (Da)	Yield (%)
UPyGGG-MPAL	**P7**	541.2	540.6	88% (97 mg)
UPyGGGCGGK(UPy)G	**P9**	1035.3	1036.0	*n.i.**
$[UPyGGGC]_4$-dendrimer	**D1P7**	2017.3	2018.2	*n.i.**
$[UPyGGGC]_1[AcGRGDSGGC]_3$-dendrimer	**D1P3P7**	2936.2	2937.1	*n.i.**

*not isolated

On an analytical scale the biohybrid thioester **P7** was successfully ligated to linear as well as dendritic constructs and analyzed on RP-HPLC. No products were purified or studied for their biological use.

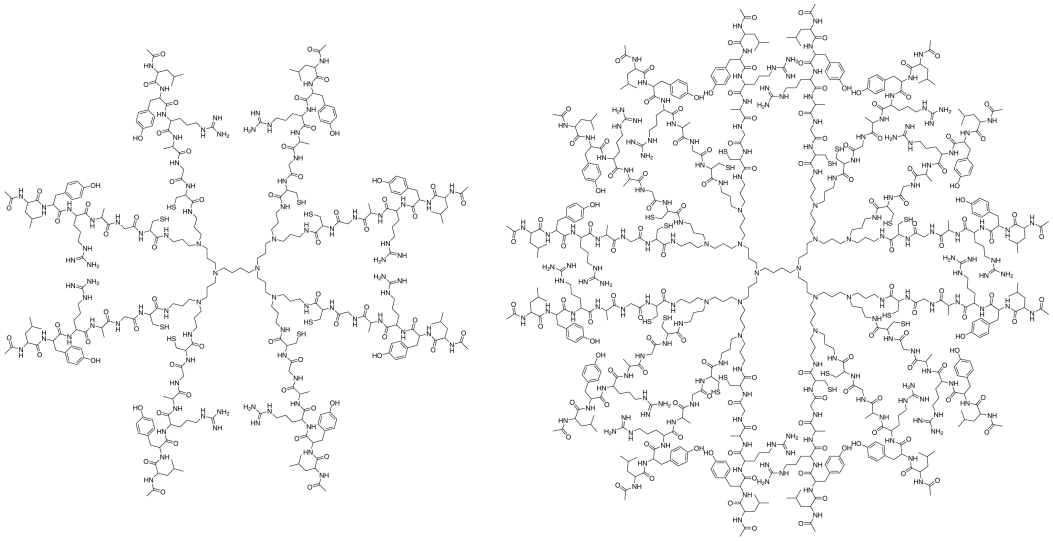
3.3.5 CONCLUSIONS

In summary, various water-soluble peptides and biohybrids were successfully attached to first generation cysteine dendrimers **D1**. By optimization of reaction conditions non-water-soluble peptides could also be ligated. Besides the coupling of four identical peptides to one dendrimer, different peptides could be coupled, resulting in a mixture of peptide dendrimer products. Isolated peptide dendrimers were obtained in good yields using RP-HPLC. These findings illustrate the high potential of native chemical ligation for use in the synthesis of first generation peptide dendrimers. As no major limitations were observed in the synthesis

of first generation peptide dendrimers, the technique was applied to the synthesis of higher generation peptide dendrimers.

3.4. LIGATION TO HIGHER GENERATION CYSTEINE DENDRIMERS

Ligation of short oligo-peptides to higher generation cysteine dendrimers (**D2** and **D3**) is expected to be more complicated compared to first-generation cysteine dendrimers since crowding might affect the ligation efficiency. As the dendrimer generation increases, the local density of end groups becomes higher (Figure 3.16). Here the ligation of oligo-peptides to the second (section 3.4.1) and third generation cysteine dendrimer (section 3.4.2) is described.



D2P2 **D3P2**

Figure 3.16 *Structural formula of [AcLYRAGC]$_8$-dendrimer **D2P2** and [AcLYRAGC]$_{16}$-dendrimer **D3P2**.*

3.4.1 LIGATION TO SECOND GENERATION CYSTEINE DENDRIMERS

Ligation of **D2** with oligo-peptides **P1-P4** was done using standard ligation conditions (10 mg/mL peptides, 2% thiophenol, 2% benzylmercaptan, Tris buffer containing 6 M guanidine, pH 7, 37 °C) and after one hour the reaction was analyzed by RP-HPLC. The product peaks in the RP-HPLC chromatograms were broader and the ESI-MS spectra were noisier compared to the peaks of the first generation peptide dendrimers (Figure 3.17). In the *m/z* spectrum for **D2P2** peaks were visible corresponding to the product $[M+10H]^{10+}$ = 643 Da; $[M+9H]^{9+}$ = 714 Da; $[M+8H]^{8+}$ = 803 Da; $[M+7H]^{7+}$ = 918 Da; $[M+6H]^{6+}$ = 1070 Da; $[M+5H]^{5+}$ = 1284 Da and $[M+4H]^{4+}$ = 1605 Da. After deconvolution, the observed mass (6419.7 Da) corresponded to the calculated mass (6419.9 Da), indicating that the desired product **D2P2** was formed.

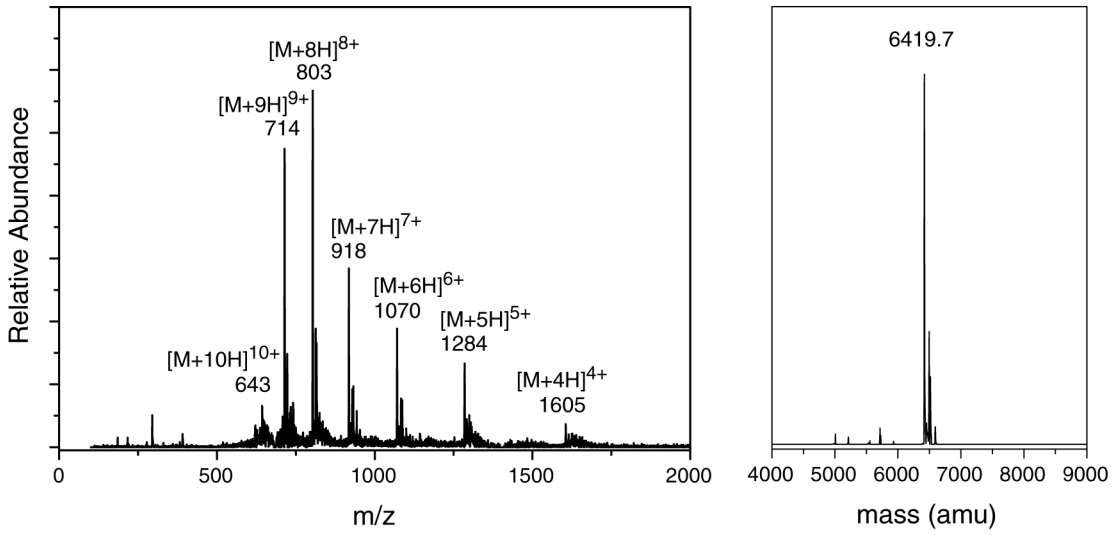


Figure 3.17. *ESI-MS spectrum and corresponding deconvoluted spectrum of [AcLYRAGC]$_8$-dendrimer* ***D2P2*** *(calculated mass: 6419.9 Da).*

In the *m/z* spectrum of [AcGGC]$_8$-dendrimer **D2P1**, peaks are shown corresponding to $[M+4H]^{4+}$ = 712 Da; $[M+3H]^{3+}$ = 949 Da and $[M+2H]^{2+}$ = 1424 Da. After deconvolution the found mass (2847.3 Da) corresponded to the calculated mass (2847.5 Da). The *m/z* spectrum of [AcGRGDSGGC]$_8$-dendrimer **D2P3** showed peaks corresponding to $[M+9H]^{9+}$ = 737 Da; $[M+8H]^{8+}$ = 829 Da; $[M+7H]^{7+}$ = 947 Da; $[M+6H]^{6+}$ = 1105 Da; $[M+5H]^{5+}$ = 1326 Da and $[M+4H]^{4+}$ = 1658 Da. After deconvolution the found mass (6627.1 Da) corresponded to the calculated mass (6627.2 Da) and the product could be isolated in 23% yield using RP-HPLC.

The ligation of AcKLVFFGG-MPAL **P5** to **D2** showed the same trend as the ligation of **P5** to the first generation cysteine dendrimer **D1**. The thioester-peptide had a low solubility in aqueous media and therefore, the reaction did not go to completion. As a consequence, all intermediate products were seen on RP-HPLC and ESI-MS (experimental section). This problem was solved by adding 10% of DMF to the reaction mixture. As a result the thioester-peptide was soluble and the reaction went to completion. In the *m/z* spectrum peaks are shown corresponding to $[M+10H]^{10+}$ = 793 Da; $[M+9H]^{9+}$ = 881 Da; $[M+8H]^{8+}$ = 991 Da; $[M+7H]^{7+}$ = 1133 Da; $[M+6H]^{6+}$ = 1321 Da; $[M+5H]^{5+}$ = 1585 Da and $[M+4H]^{4+}$ = 1981 Da. After deconvolution, the mass found (7924.8 Da) corresponded to the calculated mass (7926.1 Da).

Table 3.7 *Calculated and observed mass of several second generation peptide-dendrimers.*

Peptide-dendrimer		Obs. mass (Da)	Calc. mass (Da)	Yield (%)
[AcGGC]$_8$-dendrimer	**D2P1**	2847.3	2847.5	*n.i.**
[AcLYRAGC]$_8$-dendrimer	**D2P2**	6419.7	6419.9	*n.i.**
[AcGRGDSGGC]$_8$-dendrimer	**D2P3**	6627.1	6627.2	23% (4.5 mg)
[AcPHSRNGGC]$_8$-dendrimer	**D2P4**	-	7580.6	*n.i.**
[AcKLVFFGGC]$_8$-dendrimer	**D2P5**	7924.8	7926.1	*n.i.**

*not isolated

Ligation of thioester peptides **P1-P3** to the second generation cysteine dendrimer **D2** was successful and peptide-dendrimer products could be isolated. In the ligation of **P4** to **D2** complete conversions of the starting materials was observed with RP-HPLC, but the product peak could not be analyzed with ESI-MS. Similar to the ligation of **D1**, **P5** could be successfully ligated to **D2** using an organic cosolvent for solubility reasons.

3.4.2 LIGATION TO THIRD GENERATION CYSTEINE DENDRIMERS

As ligation to the second generation cysteine dendrimer **D2** was successful, the coupling to third generation dendrimers was also explored. The thioester-peptides (**P1-P4**) were used for ligation to the third generation cysteine dendrimer **D3**. In an attempt, the thioester-peptide **P2** without an *N*-terminal acetyl group (**P2'**, 16 equivalents) was added to **D3** (1 equivalent) in a Tris buffer of at pH 7-7.5 containing 6 M guanidine and 2 v% of thiophenol and 2 v% benzylmercaptan. After 1 hour, the chromatogram of RP-HPLC showed full conversion of the bare dendrimer **D3** and a broad hump that could correspond to the product, but could not be analyzed with ESI-MS. The mixture was left for an additional week, after which a similar pattern on HPLC was observed. Then, the reaction mixture was washed with diethyl ether and dialyzed for 3 days followed by freeze-drying. The obtained fluffy white powder was analyzed with MALDI-TOF-MS, and a very noisy spectrum was observed (see experimental section). It showed a peak consistent with the formation of $[LYRAGC]_{16}$-dendrimer **D1P2'** at 12262 Da (calculated mass: 12307 Da). However, the spectrum also contained peaks corresponding to $[LYRAGC]_{15\text{-}12}$-dendrimer, indicating the ligation reaction likely worked, but did not go to completion.

Although several attempts were carried out for ligating **P1-P4** to **D3** and applying a range of different reaction conditions and work-up procedures (see experimental section) it remained a challenge to isolate the desired construct in this way. A possible explanation could be that a heterogeneous mixture was obtained by incomplete modification due to steric reasons or the incomplete reduction of thiols of the cysteine residues (Chapter 4). Another interpretation might be that only the fully modified peptide dendrimer was obtained, but that the product could not be analyzed using RP-HPLC in combination with ESI-MS. In Chapter 4, an alternative and successful approach to functionalize third generation dendrimers with peptides is described. In this case, the fully modified third generation dendrimer could be obtained and analyzed via direct injection ESI-MS. For the experiments described in this chapter, separation is necessary on an HPLC column in order to remove buffer components.

Presumably, native chemical ligation works on the third generation cysteine dendrimer, but the reaction is difficult to monitor and analyze using RP-HPLC combined with ESI-MS. Future research should focus on suitable techniques for the analysis of third generation peptide dendrimers made in this manner.

As there are still many challenges in the characterization of third generation peptide dendrimers, dendrimers of higher generations were not tested for their capability of ligating thioester-peptides.

3.5. CONCLUSIONS AND OUTLOOK

It was demonstrated that native chemical ligation was an attractive and general synthetic strategy for the modification of dendrimers with oligopeptides. For that purpose, a first, second and third generation dendrimer were synthesized having *N*-terminal cysteine residues at their periphery and several *C*-terminal thioester-peptides with different biological or chemical function have been synthesized in good yields and high purity based on HPLC purification and ESI-MS analysis.

Ligation of various water-soluble peptides and biohybrids to a first generation cysteine dendrimer succeeded for all peptides investigated. Also, non-water soluble peptides could be ligated by optimization of the reaction conditions. Besides the coupling of four identical peptides, different peptides could be coupled to one dendrimer, resulting in a mixture of peptide dendrimer conjugates. Isolated peptide dendrimers were obtained in good yields using RP-HPLC. The biological application of some of the resulting peptide dendrimers is described in Chapter 5. No major limitations were observed for ligation with the first generation.

Second generation peptide dendrimers were obtained using the same conditions as for ligation with the first generation, and the products could be isolated using RP-HPLC. Whereas the ligation of thioester-peptides to a second generation cysteine dendrimer was successful and products could be isolated, the ligation of the third generation dendrimers showed problems in the analysis of the product. Full conversion of the starting material **D3** was observed using RP-HPLC, but the product peak could not be characterized with ESI-MS. However, MALDI-TOF analysis was indicative of product formation.

The modular approach presented here provides access to a wide variety of well-defined multivalent peptides that are attractive for understanding the fundamental mechanisms of mutlivalency in biological interactions and for biomedical applications in targeted drug delivery, molecular imaging and immunology (Chapter 5).

3.6. EXPERIMENTAL SECTION

General methods

Unless stated otherwise, all solvents (*p.a.* quality) and other chemicals were obtained from commercial sources and used as received. Water was demineralized prior to use. Dichloromethane was obtained by distillation from P_2O_5. Amine-terminated poly (propylene imine) dendrimers were kindly provided by DSM (Geleen, The Netherlands) and dried prior to use. Trityl-protected cysteine (Trt-Cys(Trt)-OH) was obtained from Bachem (Bubendorf, Switserland). Standard ^{1}H NMR and ^{13}C NMR experiments were performed on a Varian Gemini-2000 300 MHz spectrometer, a Varian Mercury Vx 400 MHz spectrometer, and a Varian Unity Inova 500 MHz spectrometer at 298 K. Chemical shifts are reported in parts per million relative to tetramethylsilane (TMS).

Reversed phase high pressure liquid chromatography (RP HPLC) was performed on a Varian Pro Star HPLC system coupled to a UV-Vis detector probing at 214 nm using a VydacTM protein & peptide C18 column. MALDI-TOF spectra were obtained on a Perspective Biosystems Voyager DE-Pro mass spectrometer using α-cyano-4-hydroxycinnamic acid as matrix. ESI-MS spectra were measured on an Applied Biosystems ESI Mass Spectrometer API-150EX, a Micromass Q-TOF Ultima Global Mass Spectrometer, or a Thermo Finnigan LCQ Deca XP MAX, all in positive mode. Magtran software was used for deconvolution.

*Trt-Cys(Trt)-OSuc (**13**)*

Trt-Cys(Trt)-OH (7.0 g, 11.6 mmol, 1 eq) was dissolved in 20 mL DMF while triethylamine (2 mL, 1.4 g, 14 mmol, 1.2 eq), HBTU (4.43 g, 11.6 mmol, 1 eq) and *N*-hydroxysuccinimide (1.6 g, 14 mmol, 1.2 eq) were added. The mixture was stirred overnight at room temperature. An aqueous hydrogen chloride solution of pH 3 was added and the reaction mixture was extracted 3 times with diethyl ether. The organic layers were combined and washed 5 times with water, dried over magnesium sulfate and evaporated under reduced pressure. The yield of the off-white solid product was 89%. ^{1}H NMR ($CDCl_3$); δ (ppm); 7.12-7.52 (m, 30H, Trt), 3.72 (dt, 1H, CH), 2.67 (s, 4H, CH_2, Suc), 2.6 (d, 1H, NH), 2.5 (dd, 1H, CH_2), 2.4 (dd, 1H, CH_2). ^{13}C NMR ($CDCl_3$, 100 MHz); δ (ppm); 168.1, (2xCq, Suc), 167.7 (C=O), 144.8 (3xCq, NHTrt), 144 (3xCq, NHTrt), 129.4 (3xCq, STrt), 129.3 (3xCq, STrt), 128 (3xCH, STrt), 127.7 (3xCH, NHTrt), 127.4 (3xCH, NHTrt), 126.9 (3xCH, STrt), 126.2 (3xCH, STrt), 126.1 (3xCH, NHTrt), 70.7 (Cq, NHTrt), 66.4 (Cq, STrt), 53.4 (CH), 36.6 (CH_2), 25 (CH_2 (Suc)). Mass (ESI): calculated: 702.8, found: 725.1 ($M+Na^+$) and 1426.9 ($2M+Na^+$).

*$[C]_4$-dendrimer (**D1**)*

The first generation poly(propylene imine) dendrimer (63 mg, 0.2 mmol, 1 eq) was dissolved in 2 mL dichloromethane and Et_3N (1.4 mL, 1 mmol, 5 eq) was added. Then Trt-Cys(Trt)-OSuc **13** (618 mg, 0.88 mmol, 4.4 eq) was added and the reaction mixture was stirred for 2 hours at room temperature. The reaction mixture was washed KCO_3 (aq, sat) and $KHSO_4$ (aq, sat). The organic layer was dried over magnesium sulfate and after filtration the solvent was evaporated under reduced pressure. The obtained compound (108 mg) was kept at 0° C using ice while 10 mL of a mixture of TFA with 2.5% of Et_3SiH and 2.5% of water was added to remove the trityl protective groups. After stirring for 1 hour, water was added and the solution was washed three times with diethyl ether. The water layer was lyophilized to obtain the product. The crude product (88%, 50 mg) was purified with RP-HPLC using a gradient of 0-20% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure dendrimer was obtained as white sticky solid in 76% yield (43 mg). ^{1}H NMR (300 MHz, CD_3OD); δ (ppm); 4.01 (t, 4H, CH), 3.48 (q, 8H, CH_2NH), 3.26-3.17 (m, 12H, CH_2N), 3.09-2.94 (m, 8H, CH_2S), 1.97 (m, 8H, $NCH_2CH_2CH_2NH$), 1.81 (m, 4H, $NCH_2CH_2CH_2CH_2N$). ESI-MS: found mass: 729.4 Da, calculated mass: 729.1Da.

*$[C]_8$-dendrimer (**D2**)*

The second generation poly(propylene imine) dendrimer (31.5 mg, 0.04 mmol, 1 eq) was dissolved in 4 mL dichloromethane and Et_3N (56 μL, 0.4 mmol, 10 eq) was added. Then Trt-Cys(Trt)-OSuc **1** (252 mg, 0.36 mmol, 8.8 eq) was added and the reaction mixture was stirred for 2 hours at room temperature.

Work up procedure was performed as described with **D1**. The crude product (79%, 100 mg) was purified with RP-HPLC using a gradient of 5-25% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure dendrimer was obtained as white sticky solid in 60% yield (75 mg). ESI-MS: found mass: 1597.6 Da, calculated mass: 1598.4 Da.

*$[C]_{16}$-dendrimer (**D3**)*

The first generation poly(propylene imine) dendrimer (34.3 mg, 0.02 mmol, 1 eq) was dissolved in 4 mL dichloromethane and Et_3N (56 µL, 0.4 mmol, 20 eq) was added. Then Trt-Cys(Trt)-OSuc **1** (252 mg, 0.36 mmol, 17.6 eq) was added and the reaction mixture was stirred for 2 hours at room temperature. Work up procedure was performed as described with **D1**. The crude product (41%, 56 mg) was purified with RP-HPLC using a gradient of 10-30% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure dendrimer was obtained as white sticky solid in 28% yield (38 mg). ESI-MS: found mass: 3335.7 Da, calculated mass: 3337.1 Da.

General synthesis of MPAL-activated peptides

Standard *tert*-butyloxycarbonyl-mediated solid phase peptide synthesis was used to synthesize the thioester-peptides. For the formation of the thioester, first 1.1 mmol Boc-Leu(·H_2O) was coupled to an MBHA (4-methyl-benzhydrylamine) resin (266 mg, target scale: 0.25 mmol, resin loading: 0.94 mmol/g).[42] For this, the amino acid was first activated using 2 mL of a 0.5 M solution of *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate (HBTU) in DMF and 0.5 mL diisopropylethyl amine (DIPEA) before coupling to the resin for 30 minutes. After this, the resin was thoroughly washed and the *tert*-butyloxycarbonyl (*t*Boc) protective group was removed by reaction with trifluoroacetic acid (TFA, 2 × 1 min). Again, the resin was thoroughly washed with DMF. Subsequently, 1.1 mmol of trityl-mercaptopropionic acid (Trt-MPA) was activated with HBTU in DMF, added to the resin and allowed to couple for 30 minutes. After thoroughly washing the resin with DMF, the trityl group was removed by reaction with a mixture of TFA and 2.5% of Et_3SiH and 2.5% of water (2 × 1 min), followed by washing with DMF. Then, the first amino acid of the desired peptide sequence was activated with HBTU and coupled (depending on the amino acid 10 or 20 minutes), followed by washing with DMF, deprotection with TFA (2 × 1 min) and washing with DMF. These steps were repeated for the remaining amino acids in the peptide sequence. When the sequence is finished, the resin is thoroughly washed with subsequently DMF, dichloromethane, and a 1:1 mixture of dichloromethane and methanol. The resin is dried under reduced pressure. After cleavage of the peptide from the resin with HF and some scavengers, the product was lyophilized and purified using reversed phase high pressure liquid chromatography (RP-HPLC) and characterized using electrospray ionization mass spectrometry (ESI-MS).

*AcGG-MPAL (**P1**)*

After the synthesis of MPAL-thioester to the resin, respectively, Boc-Gly and Boc-Gly were coupled for 10 minutes each using standard coupling protocols. The *N*-terminus of the peptide sequence was acetylated using a 0.5 M solution of acetic anhydride in DMF together with a 0.5 M solution of pyridine in DMF (2 × 2 minutes). The resin was washed and dried and the peptide sequence was cleaved from the resin using the above-mentioned HF cleavage procedure. The peptide was purified using RP-HPLC using a gradient of 5-25% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 53% yield (80 mg). ESI-MS: found mass ($[M+H]^+$): 375.0 Da, calculated mass: 374.4 Da.

*AcLYRAG-MPAL (**P2**)*

After the synthesis of MPAL-thioester to the resin, respectively, Boc-Gly and Boc-Ala were coupled for 10 minutes each using standard coupling protocols; Boc-Arg(Tos) was coupled for 20 minutes; Boc-Tyr(2-Br-Z) and Boc-Leu(·H_2O) were coupled for 10 minutes. The N-terminus of the peptide sequence was acetylated using a 0.5 M solution of acetic anhydride in DMF together with a 0.5 M solution of pyridine in DMF (2 × 2 minutes). The resin was washed and dried and the peptide sequence was cleaved from the resin using the above-mentioned HF cleavage procedure. The peptide was purified using RP-HPLC using a gradient of 20-40% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 78% yield (93 mg). ^1H NMR

(500 MHz, D_2O/ACN-d^3); δ (ppm); 7.23 (t, 2H, CH Tyr), 6.93 (t, 2H, CH Tyr), 4.68 (t, 1H, CH Tyr), 4.46 (q, 1H, CH Ala), 4.39 (m, 2H, CH Leu1, Arg), 4.33 (m, 1H, CH Leu7), 4.25 (m, 2H, CH_2 Gly), 3.28 (t, 2H, CH_2 Arg), 3.25 (t, 2H, CH_2S MPA), 3.17 (m, 1H, CH_2 Tyr), 3.06 (m, 1H, CH_2 Tyr), 2.72 (t, 2H, CH_2CO MPA), 2.11 (s, 3H, CH_3 Ac), 1.91 (sep, 1H, CH Leu1), 1.47-1.82 (m, 9H, CH_2 2xArg, Leu1, Leu7; CH Leu7), 1.55 (dd, 3H, CH_3 Ala), 1.01 (dd, 12H, CH_3 Leu1 & Leu7). Peaks assigned by ^1H,^1H-COSY NMR. ESI-MS: found mass ($[M+H]^+$): 821.4 Da, calculated mass: 821.0 Da.

*AcGRGDSGG-MPAL (**P3**)*

After the synthesis of MPAL-thioester to the resin, respectively, Boc-Gly, Boc-Gly, Boc-Ser(Bzl), Boc-Asp(O*c*Hx), and Boc-Gly were coupled for 10 minutes each using standard coupling protocols; Boc-Arg(Tos) was coupled for 20 minutes; Boc-Gly was coupled for 10 minutes. The N-terminus of the peptide sequence was acetylated using a 0.5 M solution of acetic anhydride in DMF together with a 0.5 M solution of pyridine in DMF (2 × 2 minutes). The resin was washed and dried and the peptide sequence was cleaved from the resin using the above-mentioned HF cleavage procedure. The peptide was purified using RP-HPLC using a gradient of 0-25% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 84% (92 mg) yield.^1H NMR (D_2O/ACN-d^3); δ (ppm); 4.94 (t, 1H, CH, Asp), 4.58 (t, 1H, CH, Ser), 4.47 (m, 1H, CH, Arg), 4.41 (m, 1H, CH, Leu), 4.28 (s, 2H, CH_2, Gly), 4.12-3.99 (m, 8H, CH_2, Gly (3x), Ser), 3.32 (t, 2H, CH_2, Arg), 3.29 (m, 2H, CH_2, MPA), 3.04 (m, 2H, CH_2, Asp), 2.74 (t, 2H, CH_2, MPA), 2.18 (s, 3H, CH_3, Ac), 2.01-1.68 (m, 7H, CH_2, Arg (2x), Leu; CH, Leu), 1.07 (dd, 3H, CH_3, Leu), 1.02 (dd, 3H, CH_3, Leu). Peaks assigned by ^1H,^1H-COSY NMR. ESI-MS: found mass ($[M+H]^+$): 847.5 Da, calculated mass: 846.9 Da.

*AcPH(DNP)SRNGG-MPAL (**P4**)*

After the synthesis of MPAL-thioester to the resin, respectively, Boc-Gly and Boc-Gly were coupled for 10 minutes each using standard coupling protocols; Boc-Asn(xanthyl) and Boc-Arg(Tos) were coupled for 20 minutes; Boc-Ser(Bzl), Boc-His(DNP·^{i}PrOH) and Boc-Pro were coupled for 10 minutes each. The *N*-terminus of the peptide sequence was acetylated using a 0.5 M solution of acetic anhydride in DMF together with a 0.5 M solution of pyridine in DMF (2 × 2 minutes). The resin was washed and dried and the peptide sequence was cleaved from the resin using the above-mentioned HF cleavage procedure. The peptide was purified using RP-HPLC using a gradient of 15-35% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 56% yield (75 mg). ^1H NMR (500MHz, D_2O/ACN-d^3); δ (ppm); 9.33 (s, 1H, CH, DNP), 9.27 (s, 1H, CH, DNP), 8.94 (d, 0.5H, CH, His), 8.22 (d, 0.5H, CH, His), 7.8 (s, 1H, CH, DNP), 7.23 (d, 0.5H, CH, His), 6.91 (d, 0.5H, CH, His), 4.97 (t, 1H, CH, His), 4.83 (m, 1H, CH, Asn), 4.79 (m, 1H, CH, Pro), 4.6 (t, 1H, CH, Ser), 4.47 (m, 1H, CH, Arg), 4.41 (m, 1H, CH, Leu), 4.28 (s, 2H, CH_2, Gly), 4.12-3.99 (m, 4H, CH_2, Gly, Ser), 3.75 (m, 2H, CH_2, Pro), 3.59-3.25 (m, 6H, CH_2, His, Arg, MPA), 2.94 (m, 2H, CH_2, Asn), 2.73 (t, 2H, CH_2, MPA), 2.22 (s, 3H, CH_3, Ac), 2.17-1.69 (m, 9H, CH_2, Arg (2x), Leu; CH, Leu), 1.06 (dd, 3H, CH_3, Leu), 1.01 (dd, 3H, CH_3, Leu). Peaks assigned by ^1H,^1H-COSY NMR. ESI-MS: found mass ($[M+H]^+$): 1132.4 Da, calculated mass: 1132.1 Da.

*AcKLVFFGG-MPAL (**P5**)*

After the synthesis of MPAL-thioester to the resin, respectively, Boc-Gly, Boc-Gly, Boc-Phe, Boc-Phe, Boc-Val, Boc-Lys(2-Cl-Z), and Boc-Leu(·H_2O) were coupled for 10 minutes each using standard coupling protocols. The *N*-terminus of the peptide sequence was acetylated using a 0.5 M solution of acetic anhydride in DMF together with a 0.5 M solution of pyridine in DMF (2 × 2 minutes). The resin was washed and dried and the peptide sequence was cleaved from the resin using the above-mentioned HF cleavage procedure. The peptide was purified using RP-HPLC using a gradient of 30-50% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 81% yield (128 mg). ESI-MS: found mass ($[M+H]^+$): 1009.6 Da, calculated mass: 1009.2 Da.

*KLVFFGG-MPAL (**P5'**)*

After the coupling of MPAL to the resin, subsequently, Boc-Gly, Boc-Gly, Boc-Phe, Boc-Phe, Boc-Val, Boc-Leu and Boc-Lys(2-Cl-Z) were coupled for 10 minutes each. The resin was washed and dried and

the peptide was cleaved using HF. The peptide was purified using RP-HPLC using a gradient of 25-45% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 58% yield (50.6 mg). ESI-MS: found mass: 967.5 Da; calculated mass: 967.2 Da.

b**enzyl 2-((4-nitrophenoxy)carbonylamino)acetate **15
Carbamate **15** was kindly provided by B.F.M. de Waal.

*clickCANPNAA-MPAL (**P6**)*
After the synthesis of MPAL-thioester to the resin, respectively, Boc-Ala and Boc-Ala were coupled for 10 minutes each using standard coupling protocols; Boc-Asn(xanthyl) was coupled for 20 minutes; Boc-Pro was coupled for 10 minutes, Boc-Asn(xanthyl) was coupled for 20 minutes; Boc-Ala and Boc-Cys(4-Me-Bzl) were coupled for 10 minutes. After deprotection of the *t*Boc groups of the final cysteine residue, the N-terminus was modified with a urea-glycine tail by coupling of carbamate **3** (2.2 mmol in 4 mL DMF and 1 mL DIPEA) for 30 minutes. The resin was washed and dried and the peptide sequence was cleaved from the resin using the above-mentioned HF cleavage procedure. The peptide was purified using RP-HPLC using a gradient of 10-30% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 36% yield (48 mg). ESI-MS: found mass ($[M+H]^+$): 961.3 Da, calculated mass: 961.0 Da.

*UPyGGG-MPAL (**P7**)*
After the synthesis of MPAL-thioester to the resin, respectively, Boc-Gly, Boc-Gly, and Boc-Gly were coupled for 10 minutes each using standard coupling protocols. CDI-activated UPy **4** (3.3 mmol) was dissolved in 6 ml DMF and 1.5 mL DIPEA (a milky solution was obtained) and coupled to the resin for 2 × 30 minutes. The resin was thoroughly washed subsequently with a 0.1 M HCl solution, DMF, dichloromethane and a 1:1 mixture of dichloromethane and methanol. The resin was dried under reduced pressure and the peptide sequence was cleaved from the resin using the above-mentioned HF cleavage procedure. The peptide was purified using RP-HPLC using a gradient of 10-30% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 88% yield (97.3 mg). ESI-MS: found mass ($[M+H]^+$): 541.2 Da, calculated mass: 540.6 Da.

*CGGK(UPy)G-MPAL (**P8**)*
P8 was kindly provided by P.Y.W. Dankers.

*$[AcGGC]_4$-dendrimer (**D1P1**)*
The peptide AcGG-MPAL (16.4 mg, 4.3×10^{-2} mmol, 4 eq) was dissolved in 500 μL of Tris buffer pH 8.0 containing 6 M guanidine and $[C]_4$-dendrimer (8 mg, 1.1×10^{-2} mmol, 1 eq) was added. Thiophenol (2 v%, 10 μL) and benzylmercaptan (2 v%, 10 μL) were added, the pH is checked (7), the mixture was vigorously stirred and put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was purified using RP-HPLC using a gradient of 5-25% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained as white sticky solid in 67% yield (12.5 mg). ESI-MS: found deconvoluted mass: 1353.0 Da, calculated mass: 1353.6 Da.

*$[AcLYRAGC]_4$-dendrimer (**D1P2**)*
The reaction was done on an analytical scale. The peptide AcLYRAG-MPAL (5.7 mg, 6.9 μmol, 5 eq) is dissolved in 550 μL of Tris buffer pH 8.0 containing 6 M guanidine and $[C]_4$-dendrimer (1 mg, 1.3 μmol, 1 eq) was added. Thiophenol (2 v%, 11 μL) and benzylmercaptan (2 v%, 11 μL) are added, the pH is checked (7), the mixture was vigorously stirred and put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was analyzed using RP-HPLC combined with ESI-MS. ESI-MS: found deconvoluted mass: 3139.7 Da, calculated mass: 3139.8 Da.

*$[AcGRGDSGGC]_4$-dendrimer (**D1P3**)*
The peptide AcGRGDSGG-MPAL (53 mg, 6.2×10^{-2} mmol, 4 eq) is dissolved in 2.5 mL of Tris buffer pH 8.0 containing 6 M guanidine and $[C]_4$-dendrimer (11 mg, 1.5×10^{-2} mmol, 1 eq) was added. Thiophenol (2 v%, 50 μL) and benzylmercaptan (2 v%, 50 μL) are added, the pH is checked (6) and adjusted to 7.5 with 10 μL of a 5 M solution of sodium hydroxide in water. The mixture was vigorously stirred and

put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was purified using RP-HPLC using a gradient of 5-25% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The synthesis was done several times and the pure peptide was obtained in yields varying from 36-53% (8.9-27.8 mg). ESI-MS: found deconvoluted mass: 3243.2 Da, calculated mass: 3243.5 Da.

*$[AcPHSRNGGC]_4$-dendrimer (**D1P4**)*

The reaction was done on an analytical scale. The peptide AcPHSRNGG-MPAL (7.0 mg, 6.1 μmol, 4.5 eq) is dissolved in 500 μL of Tris buffer pH 8.0 containing 6 M guanidine and $[C]_4$-dendrimer (1 mg, 1.3 μmol, 1 eq) was added. Thiophenol (2 v%, 10 μL) and benzylmercaptan (2 v%, 10 μL) are added, the pH is checked (7), the mixture was vigorously stirred and put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was analyzed using RP-HPLC combined with ESI-MS. ESI-MS: found deconvoluted mass: 3720.4 Da, calculated mass: 3720.1 Da.

*$[AcKLVFFGGC]_4$-dendrimer (**D1P5**)*

Analytical scale: The peptide AcKLVFFGG-MPAL (3.1 mg, 3 μmol, 4.5 eq) is dissolved in 100 μL of Tris buffer pH 8.0 containing 6 M guanidine and 10 μL of a co-solvent (DMF, DMSO, or acetonitrile). $[C]_4$-dendrimer (0.5 mg, 6.8×10^{-4} mmol, 1 eq) was added and thiophenol (2 v%, 2 μL) and benzylmercaptan (2 v%, 2 μL) were added. The pH is checked (7.5), the mixture was vigorously stirred and put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was analyzed using RP-HPLC combined with ESI-MS. Only the fully converted dendrimer ($[AcKLVFFGGC]_4$-dendrimer) was found. ESI-MS: found deconvoluted mass: 3892.3 Da, calculated mass: 3892.9 Da. Using the same conditions, but without organic co-solvent, all intermediate products were found. ESI-MS: $[AcKLVFFGGC]_4$-dendrimer **D1(P5)$_4$**; found deconvoluted mass: 3892.3 Da, calculated mass: 3892.9 Da; **D1(P5)$_3$**; found deconvoluted mass: 3101.3 Da, calculated mass: 3101.9 Da; **D1(P5)$_2$**; found deconvoluted mass: 2310.9 Da, calculated mass: 2311.0 Da; **D1(P5)$_1$**; found deconvoluted mass: 1519.6 Da, calculated mass: 1520.0 Da.

Preparative scale: The peptide AcKLVFFGG-MPAL (40 mg, 3.9×10^{-2} mmol, 4 eq) is dissolved in 460 μL of Tris buffer pH 8.0 containing 6 M guanidine and 40 μL of DMF was added. $[C]_4$-dendrimer (7.2mg, 9.9 μmol, 1 eq) was added. Thiophenol (2 v%, 10 μL) and benzylmercaptan (2 v%, 10 μL) are added, the pH is checked (7), the mixture was vigorously stirred and put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was purified using RP-HPLC using a gradient of 35-55% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide-dendrimer was obtained in 8% yield (3 mg).

*$[KLVFFGGC]_4$-dendrimer (**D1P5'**)*

$[C]_4$-dendrimer **D1** (9.3 mg, 1.2×10^{-2} mmol, 1 eq) was dissolved in 2 mL Tris buffer pH 8.0 containing 6 M guanidine buffer and KLVFFGG-MPAL **P5'** (49.1 mg, 5.0×10^{-2} mmol, 4 eq) was added. Thiophenol (2 v%, 40 μL) and benzylmercaptan (2 v%, 40 μL) were added, the pH was checked (7.5) and the mixture was vigorously stirred. After 1 hour of reaction time, the peptide-dendrimer was purified using RP-HPLC using a gradient of 30-50% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide-dendrimer was obtained in 25% yield (12.2 mg). ESI-MS: found mass: 3725.2 Da; calculated mass: 3724.8 Da.

*$[clickCANPNAAC]_4$-dendrimer (**D1P6**)*

$[C]_4$-dendrimer (9.1 mg, 1.2×10^{-2} mmol, 1 eq) was dissolved in 1.2 mL Tris buffer pH 8.0 containing 6 M guanidine and clickCANPNAA-MPAL (48 mg, 5.0×10^{-2} mmol, 4 eq) was added. Thiophenol (2 v%, 25 μL) and benzylmercaptan (2 v%, 25 μL) were added, the pH was checked (7) and the mixture was vigorously stirred. After 1 hour of reaction time, the peptide-dendrimer was purified using RP-HPLC using a gradient of 10-30% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide-dendrimer was obtained in 20% yield (9 mg). ESI-MS: found mass: 3694.8 Da; calculated mass: 3700.1 Da.

*UPyGGGCGGK(UPy)G (**P9**)*

The reaction was done on an analytical scale. The peptide UPyGGG-MPAL **P7** (0.5 mg, 9.2×10^{-4} mmol, 1 eq) is dissolved in 50 μL of Tris buffer pH 8.0 containing 6 M guanidine and CGGK(UPy)G **P8** (0.66 mg, 9.2×10^{-4} mmol, 1 eq) was added. Thiophenol (2 v%, 1 μL) and benzylmercaptan (2 v%, 1 μL) are added, the pH is checked and adjusted with a 1 M solution of sodium hydroxide to 7.5, the mixture was vigorously stirred and put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was analyzed using RP-HPLC combined with ESI-MS. ESI-MS: found deconvoluted mass: 1035.3 Da, calculated mass: 1036.0 Da.

*$[UPyGGGC]_4$-dendrimer (**D1P7**)*

The reaction was done on an analytical scale. The peptide UPyGGG-MPAL (1.5 mg, 2.7 μmol, 4 eq) is dissolved in 100 μ of Tris buffer pH 8.0 containing 6 M guanidine and $[C]_4$-dendrimer (0.5 mg, 6.9×10^{-4} mmol, 1 eq) was added. Thiophenol (2 v%, 2 μL) and benzylmercaptan (2 v%, 2 μL) are added, the pH is checked (7.5), the mixture was vigorously stirred and put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was analyzed using RP-HPLC combined with ESI-MS. ESI-MS: found deconvoluted mass: 2017.3 Da, calculated mass: 2018.2 Da.

*$[UPyGGGC]_1[AcGRGDSGGC]_3$-dendrimer (**D1P3P7**)*

The reaction was done on an analytical scale. The peptide UPyGGG-MPAL (0.21 mg, 3.9 μmol, 1 eq) is dissolved in 20 μL of Tris buffer pH 8.0 containing 6 M guanidine and $[AcGRGDSGGC]_3[C]_4$-dendrimer (1 mg, 3.8 μmol, 1 eq) was added. Thiophenol (2 v%, 1 μL) and benzylmercaptan (2 v%, 1 μL) are added, the pH is checked (7), the mixture was vigorously stirred and put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was analyzed using RP-HPLC combined with ESI-MS. ESI-MS: found deconvoluted mass: 2936.2 Da, calculated mass: 2937.1 Da.

*$[AcGGC]_8$-dendrimer (**D2P1**)*

The reaction was done on an analytical scale. The peptide AcGG-MPAL (1.9 mg, 5 μmol, 8 eq) is dissolved in 100 μL of Tris buffer pH 8.0 containing 6 M guanidine and $[C]_8$-dendrimer (1 mg, 6.3×10^{-4} mmol, 1 eq) was added. Thiophenol (2 v%, 2 μL) and benzylmercaptan (2 v%, 2 μL) are added, the pH is checked (7.5), the mixture was vigorously stirred and put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was analyzed using RP-HPLC combined with ESI-MS. ESI-MS: found deconvoluted mass: 2847.3 Da, calculated mass: 2847.5 Da.

*$[AcLYRAGC]_8$-dendrimer (**D2P2**)*

The reaction was done on an analytical scale. The peptide AcLYRAG-MPAL (5.2 mg, 6.3 μmol, 10 eq) is dissolved in 500 μL of Tris buffer pH 8.0 containing 6 M guanidine and $[C]_8$-dendrimer (1 mg, 6.3×10^{-4} mmol, 1 eq) was added. Thiophenol (2 v%, 10 μL) and benzylmercaptan (2 v%, 10 μL) are added, the pH is checked (7), the mixture was vigorously stirred and put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was analyzed using RP-HPLC combined with ESI-MS. ESI-MS: found deconvoluted mass: 6419.7 Da, calculated mass: 6419.9 Da.

*$[AcGRGDSGGC]_8$-dendrimer (**D2P3**)*

Analytical scale: the peptide AcGRGDSGG-MPAL (2.1 mg, 2.4 μmol, 8 eq) is dissolved in 200 μL of Tris buffer pH 8.0 containing 6 M guanidine and $[C]_8$-dendrimer (0.5 mg, 3.1×10^{-4} mmol, 1 eq) was added. Thiophenol (2 v%, 4 μL) and benzylmercaptan (2 v%, 4 μL) are added, the pH is checked (7), the mixture was vigorously stirred and put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was analyzed using RP-HPLC combined with ESI-MS. ESI-MS: found deconvoluted mass: 6627.1 Da, calculated mass: 6627.2 Da.

Preparative scale: $[C]_8$-dendrimer **D2** (4.7 mg, 2.9 μmol, 1 eq) was dissolved in 1 mL Tris buffer pH 8.0 containing 6 M guanidine buffer and AcGRGDSGG-MPAL (20.9 mg, 2.4×10^{-2} mmol, 8 eq) was added. Thiophenol (2 v%, 20 μL) and benzylmercaptan (2 v%, 20 μL) were added, the pH was checked (7) and the mixture was vigorously stirred and monitored using RP-HPLC. After 1 hour of reaction time, the peptide-dendrimer was washed with ether, freeze-dried and subsequently dialysed for 3 days. After freeze-drying, the pure peptide-dendrimer was obtained in 23% yield (4.5 mg). ESI-MS: found mass: 6627.1 Da; calculated mass: 6627.2 Da

*[AcPHSRNGGC]$_8$-dendrimer (**D2P4**)*

The reaction was done on an analytical scale. The peptide AcPHSRNGG-MPAL (5.7 mg, 5.0 μmol, 8 eq) is dissolved in 500 μL of Tris buffer pH 8.0 containing 6 M guanidine and [C]$_8$-dendrimer (1 mg, 6.3×10^{-4} mmol, 1 eq) was added. Thiophenol (2 v%, 10 μL) and benzylmercaptan (2 v%, 10 μL) are added, the pH is checked (7), the mixture was vigorously stirred and put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was analyzed using RP-HPLC combined with ESI-MS. ESI-MS: product not found.

*[AcKLVFFGGC]$_8$-dendrimer (**D2P5**)*

The reaction was done on an analytical scale. The peptide AcKLVFFGG-MPAL (5.0 mg, 5 μmol, 8 eq) is dissolved in 100 μL of Tris buffer pH 8.0 containing 6 M guanidine and [C]$_8$-dendrimer (1 mg, 6.3×10^{-4} mmol, 1 eq) was added. Thiophenol (2 v%, 2 μL) and benzylmercaptan (2 v%, 2 μL) are added, the pH is checked (7.5), the mixture was vigorously stirred and put at 37 °C. After 1 hour of reaction time, the peptide-dendrimer was analyzed using RP-HPLC combined with ESI-MS. ESI-MS: [AcKLVFFGGC]$_8$-dendrimer (**D2(P5)$_8$**): found deconvoluted mass: 7924.8 Da, calculated mass: 7926.1 Da; **D2(P5)$_7$**: found deconvoluted mass: 7134.2 Da, calculated mass: 7135.1 Da; **D2(P5)$_6$**: found deconvoluted mass: 6343.9 Da, calculated mass: 6344.2 Da; **D2(P5)$_5$**: found deconvoluted mass: 5553.3 Da, calculated mass: 5553.2 Da; **D2(P5)$_4$**: found deconvoluted mass: 4761.6 Da, calculated mass: 4762.3 Da; **D2(P5)$_3$**: found deconvoluted mass: 3970.2 Da, calculated mass: 3971.3 Da **D2(P5)$_2$**: found deconvoluted mass: 3179.2 Da, calculated mass: 3180.3 Da; **D2(P5)$_1$**: found deconvoluted mass: 2387.9 Da, calculated mass: 2389.4 Da.

*[LYRAGC]$_{16}$-dendrimer **D3P2'***

The third generation cysteine dendrimer ([C]$_{16}$-dendrimer **D3** (4.5 mg, 1.3 μmol, 1 eq) was dissolved in 1 mL of Tris buffer pH 8.0 containing 6 M guanidine and added to the thioester-peptide LYRAG-MPAL **P2'** (17 mg, 0.02 mmol, 16 eq). Thiophenol (2 v%, 20 μL) and benzylmercaptan (2 v%, 20 μL) were added, the pH was checked (pH 7.5). The solution was vigorously stirred and put at 37 °C. After 1 hour, the chromatogram of RP-HPLC showed full conversion of **D3** and a broad hump that could correspond to the product. However, when analyzing this hump using ESI-MS, a very noisy spectrum was obtained. Also, when analyzing this sample with MALDI-TOF, no product was found. The reaction mixture was reacted for another week, but no differences in the chromatogram were observed. Then, the reaction mixture was washed with diethyl ether to remove excess of thiols and dialysis against water was performed (MWCO 5000 Da). After 3 days the water was freeze-dried and the obtained white fluffy solid was analyzed using MALDI-TOF. A very noisy spectrum was obtained, it showed a peak consistent with the formation of [LYRAGC]$_{16}$-dendrimer at 12262 Da (calculated mass: 12307 Da). However, the spectrum also contained peaks attributable to [LYRAGC]$_{15\text{-}12}$-dendrimer, indicating the ligation reaction works, but did not go to completion.

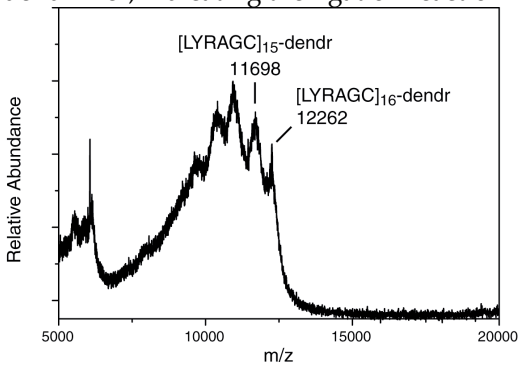


Figure 3.18 *MALDI-TOF spectrum obtained after ligation of LYRAG-MPAL with [C]$_{16}$-dendrimer **D3**. The spectrum indicates that the ligation doesn't go to completion, as intermediate products are also shown (calculated mass [LYRAGC]$_{16}$-dendrimer: 12307 Da; calculated mass [LYRAGC]$_{15}$-dendrimer: 11746 Da.*

Attempts were made to optimize the reaction conditions and work-up procedures (longer reaction times, higher temperatures, large excess of thioester-peptide), but none of these seemed to improve the reaction. Similar results were obtained for the other thioester-peptides (**P1**, **P2** and **P3**).

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CHAPTER 4

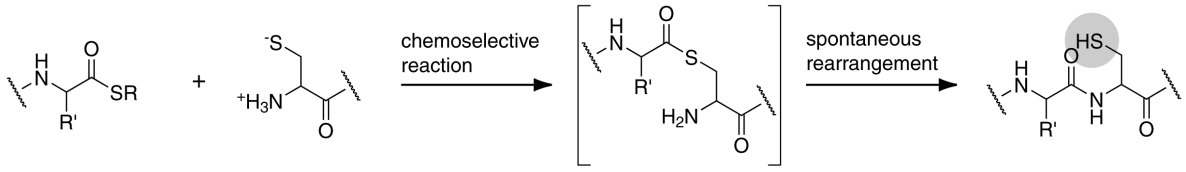
MODIFICATION OF PEPTIDE DENDRIMERS

ABSTRACT

The introduction of peptides on dendrimers using the native chemical ligation technique results in a pendant thiol group. This chapter describes the analysis and characterization of the thiol groups and their conversion into disulfide bonds, both intramolecularly and intermolecularly. The presence of thiol groups after ligation on the dendrimers can be used as a tool to incorporate additional functionalities at a later stage through reaction with maleimides. A general strategy is reported for the introduction of multiple MRI labels and fluorescent labels for optical imaging to peptide-dendrimer scaffolds, utilizing highly efficient chemoselective reaction procedures and resulting in well-defined multivalent dendritic MRI contrast agents. Finally, oxime chemistry is used in combination with maleimide-thiol reactions to develop a potential dynamic drug delivery system based on peptide dendrimer scaffolds.

4.1. REACTIVITY OF THIOL GROUPS

The introduction of peptides on dendrimers using native chemical ligation results in a pendant thiol group (Scheme 4.1). The interconversion between dithiol and disulfide is of importance for the efficiency of the ligation reaction as the thiol participates in the reaction. Furthermore, thiol groups can be used for chemical modification of peptide dendrimers, and, in this way, probes can be efficiently introduced.



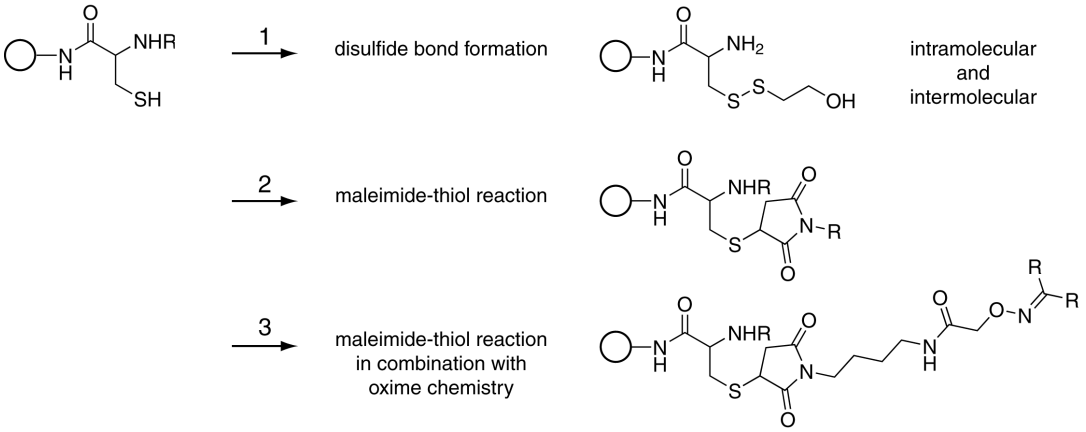
Scheme 4.1 *Native chemical ligation: the thiol of cysteine participates in the reaction, but is available again after ligation is completed.*

In proteins, thiol groups (also called mercaptans or sulfhydryls) are present in cysteine residues and the interconversion between cysteine and cystine is an important one in biochemistry. Intra-chain or inter-chain cystine (disulfide) cross-links often serve to stabilize the three-dimensional structure of proteins. For example, keratin is a structurally complex, naturally occurring protein heavily cross-linked by disulfide bonds. These disulfide bonds link individual peptide chains and form a matrix. Disulfide bonds are thus very important in maintaining the three-dimensional conformation of proteins. The formation of disulfide bonds has been studied for several oxidizing agents and varies with the agents.[1] For oxygen, it involves loss of a proton, oxidation to a free radical, and radical coupling.[2]

Thiols are easily oxidized to disulfides.[3] Hydrogen peroxide is the most common reagent,[4,5] but many oxidizing agents catalyze the reaction.[6-14] Even the oxygen in the air oxidizes thiols, if a small amount of base is present. Thiols can be generated by selectively reducing cystine disulfides with reagents such as dithiothreitol[15] (DTT) or β-mercaptoethanol, each of which must be later removed by dialysis or gel filtration before reaction with the thiol-reactive probe.[16] Unfortunately, removal of DTT or β-mercaptoethanol is often accompanied by air oxidation of the thiols back to the disulfides. Reformation of the disulfide bond can be avoided by using the reducing agent tris-(2-carboxyethylphosphine) (TCEP),[17,18] which usually does not need to be removed prior to thiol modification as it does not contain thiols; however, TCEP has been reported to react with haloacetamides or maleimides under certain conditions.[17,19,20] Thiol-reactive probes are frequently used to prepare fluorescent peptides, proteins and oligonucleotides for investigating biological structure, function and interactions.[21-25]

Here, the formation of disulfide bonds is evaluated both intramolecularly and intermolecularly (section 4.2) as only free thiols provide a tool to incorporate additional functionalities, such as fluorescent or MRI probes. A well-known synthetic approach for the

functionalization of thiols is through reaction with maleimides. This chemical modification of peptide dendrimers is assessed in section 4.3. Using the maleimide-thiol reaction it should also be possible to introduce dynamic covalent bonds, such as oxims. In this way a potential dynamic drug delivery system based on multivalent peptide dendrimers can be developed (section 4.4).



Scheme 4.2 *Overview of possible reactions described in this chapter for chemical modification of peptide dendrimers.*

4.2. CHARACTERIZATION OF DISULFIDE BRIDGES

The occurrence of both intramolecular (section 4.2.1) and intermolecular (section 4.2.2) disulfide bridges in the first, second and third generation cysteine-dendrimers **D1-D3** was investigated. This study serves as a proof of principle; all experiments were carried out on an analytical scale and analyzed with electrospray ionization mass spectrometry (ESI-MS).

4.2.1 INTRAMOLECULAR DISULFIDE BRIDGES

The dendrimer was dissolved in a volatile buffer with a mild reducing agent β-mercaptoethanol (BME). Analysis with ESI-MS revealed a spectrum corresponding to the fully reduced dendrimer (found mass: 729.4 Da; calculated mass: 729.1 Da) (Figure 4.2). Subsequently, hydrogen peroxide was added and the sample was measured again using ESI-MS. Now, a mass of 725.6 Da was found corresponding to the two isomers of the fully oxidized dendrimer (calculated mass: 725.0 Da).

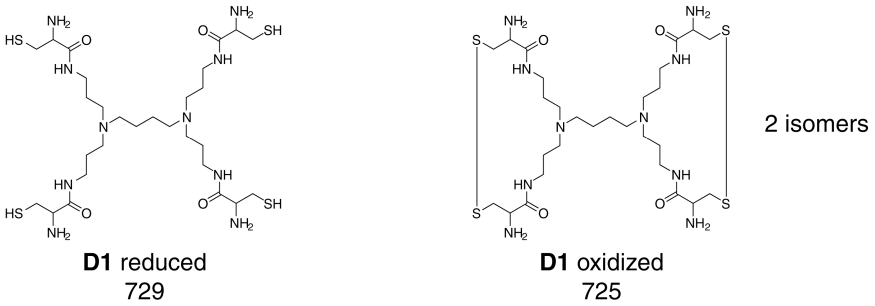


Figure 4.1 *Structural formula of the reduced and oxidized forms of **D1**. **D1** fully reduced has a calculated mass of 729.1 Da; **D1** fully oxidized has a calculated mass of 725.0 Da.*

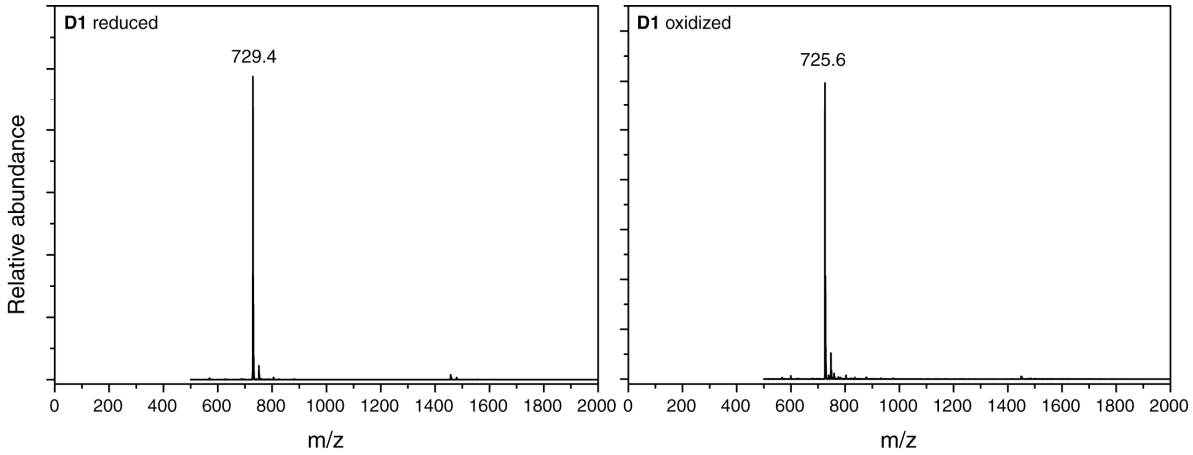


Figure 4.2 *ESI-MS spectra of **D1** under reducing conditions in a buffer at pH 2 and 0.1% of β-mercaptoethanol (left); under oxidizing conditions in a buffer at pH 8 and 0.1% of H_2O_2 (right).*

By measuring the samples in the region of 720 to 740 Da (Figure 4.3), more detailed information about the interconversion of cysteine to cystine was obtained. Again, under reducing conditions, the isotopic pattern of the fully reduced dendrimer was seen and, conversely, under oxidizing conditions the isotopic pattern of the fully oxidized dendrimer was observed. As expected, under physiological conditions, an equilibrium was reached between the free thiols and disulfide bonds; a mixture was obtained of the fully reduced dendrimer at 729 Da, the fully oxidized dendrimer at 725 Da, and the dendrimer with only one disulfide bridge and two free thiols at 727 Da (**D1** with one disulfide bridge has a calculated mass of 727.0 Da) (Figure 4.3).

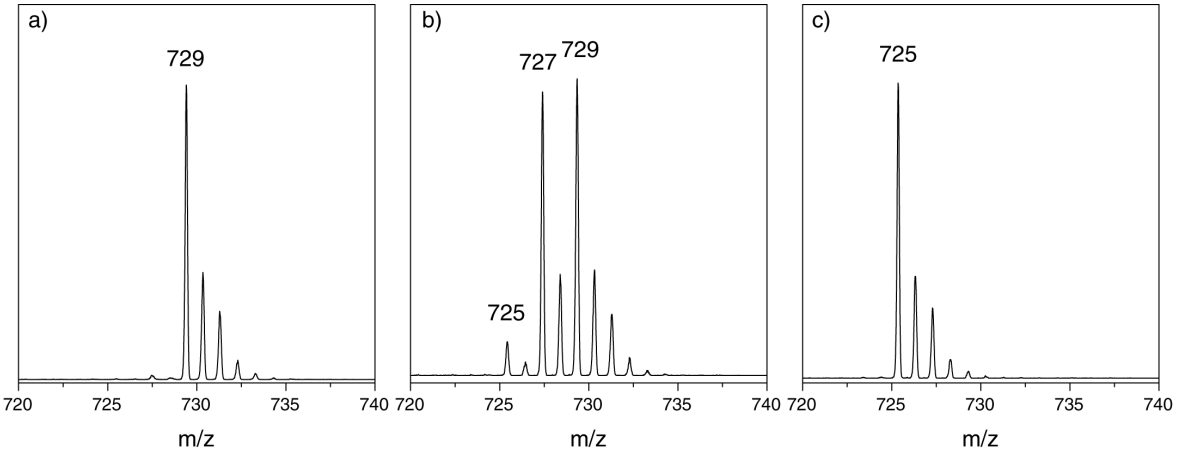


Figure 4.3 *ESI-MS spectra of **D1** measured in the region between 720 and 740 Da. The isotopic pattern is seen. a) **D1** dissolved in a buffer at pH 2 and 0.1% of BME; b) **D1** in a buffer at pH 8; c) **D1** in a buffer at pH 8 and 0.1% of H_2O_2.*

For the second generation cysteine dendrimer **D2**, the same trend was seen. **D2** has eight cysteine end groups and thus eight thiol groups capable of forming four disulfide bonds. The mass difference between the fully reduced and fully oxidized dendrimer is 8 Da and the oxidized product consists of many isomers (Figure 4.4).

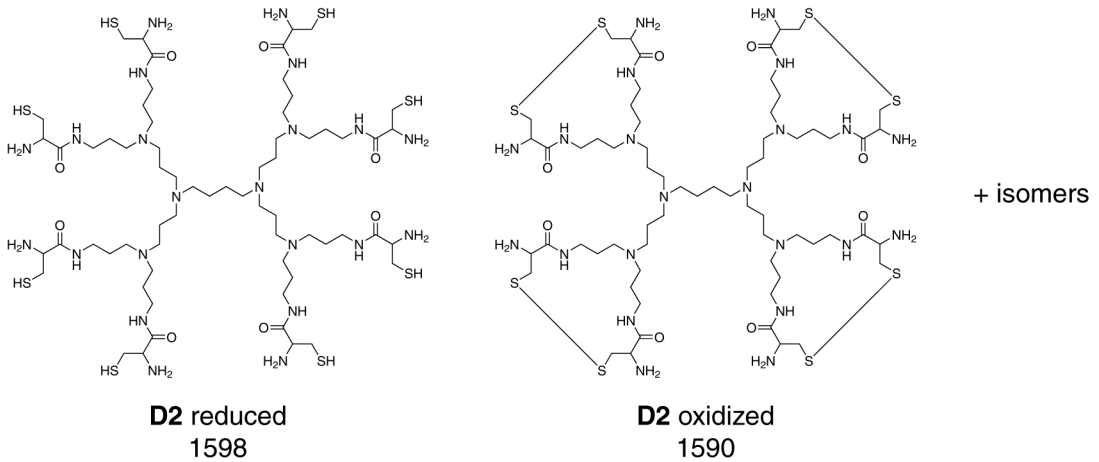


Figure 4.4 *Structural formula of the reduced and oxidized forms of **D2**. **D2** fully reduced has a calculated mass of 1598.4 Da; **D2** fully oxidized has a calculated mass of 1590.3 Da.*

The dendrimer **D2** was dissolved in a volatile buffer at pH 2 with BME. The mass spectrum shows the fully reduced dendrimer with peaks of $[M+H]^+$ at 1597 Da; $[M+2H]^{2+}$ at 799 Da and $[M+3H]^{3+}$ at 533 Da (deconvoluted mass: 1597 Da) (Figure 4.5). Again, hydrogen peroxide was added and the sample was measured. The mass spectrum shows the mass of the fully oxidized dendrimer (several isomers) with peaks of $[M+H]^+$ at 1589 Da; $[M+2H]^{2+}$ at 795 Da and $[M+3H]^{3+}$ at 531 Da (deconvoluted mass: 1589 Da).

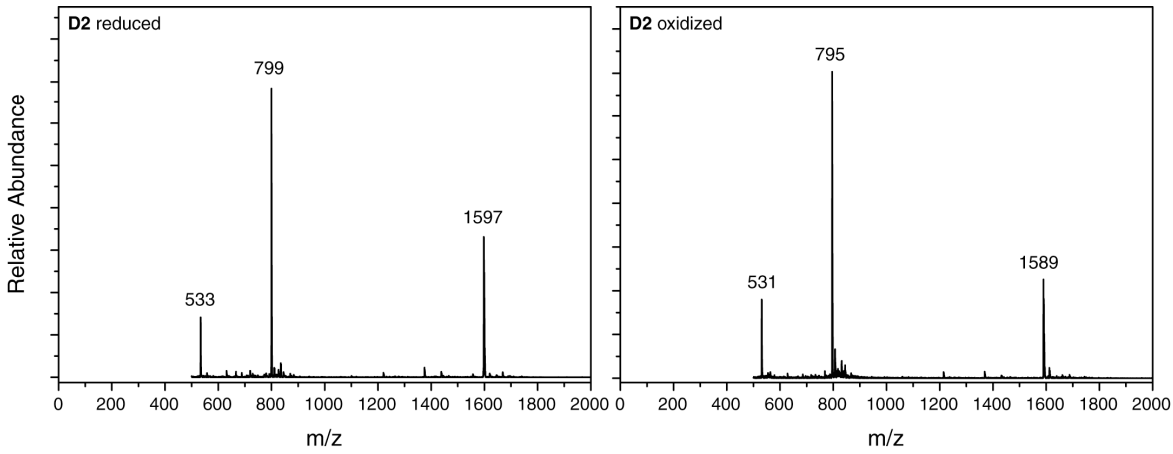


Figure 4.5 *ESI-MS spectra of* ***D2*** *under reducing conditions in a buffer at pH 2 and 0.1% of BME (left); under oxidizing conditions in a buffer at pH 8 and 0.1% of* H_2O_2 *(right).*

The third generation dendrimer **D3** has sixteen cysteine end groups, and therefore sixteen thiols capable of forming eight disulfide bonds. The mass difference between the fully reduced and fully oxidized dendrimer is thus 16 Da (Figure 4.6).

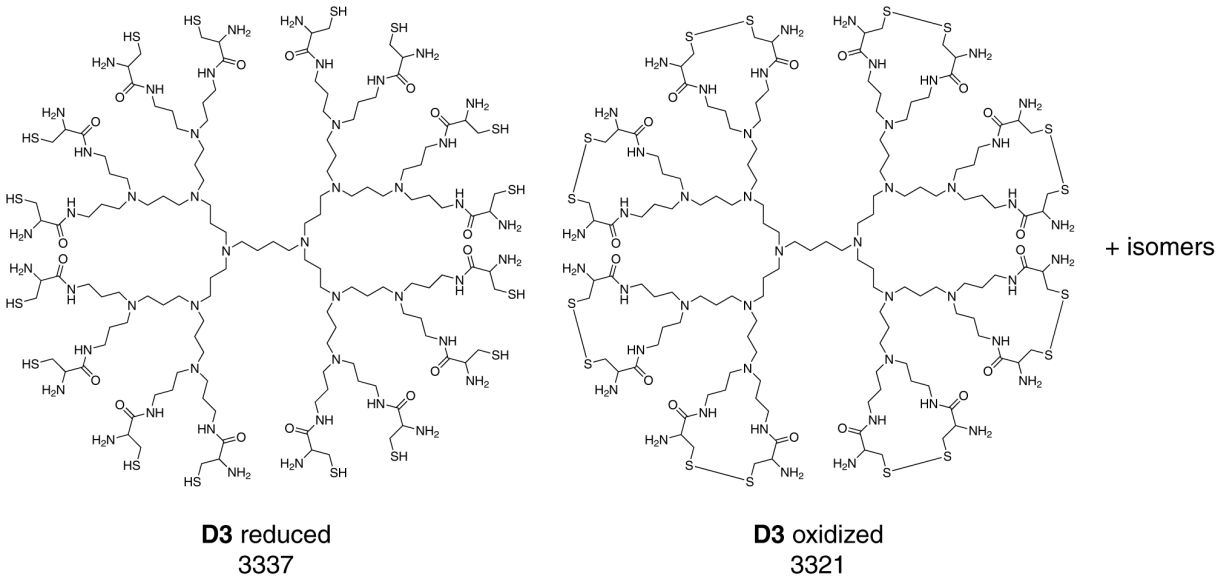


Figure 4.6 *Structural formula of the reduced and oxidized forms of* ***D3****.* ***D3*** *fully reduced has a calculated mass of 3337.1 Da;* ***D3*** *fully oxidized has a calculated mass of 3321.0 Da.*

The dendrimer **D3** was dissolved in a volatile buffer at pH 2 with BME and the sample was measured with mass spectrometry. Strikingly, a deconvoluted mass of 3335 Da was found, which corresponds to the dendrimer with one disulfide bond and 14 free thiols (calculated mass of fully reduced dendrimer: 3337 Da). Apparently, the mild reducing agent β-mercaptoethanol was either not capable of reducing all disulfide bonds, or, the thiols in the dendrimer were in such close proximity that a disulfide bond was very likely to be formed. This would also explain the observation that a heterogeneous mixture was obtained when **D3** was used in native chemical ligation (Chapter 3). When adding hydrogen peroxide, the

mass spectra showed a deconvoluted mass of 3321 Da, which corresponded to the fully oxidized dendrimer (calculated mass: 3321 Da) (Figure 4.7).

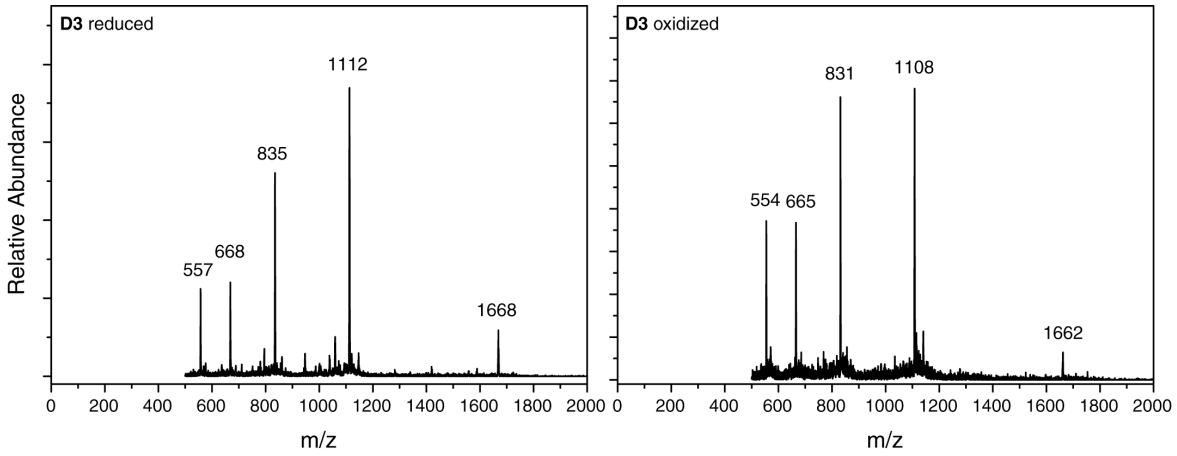
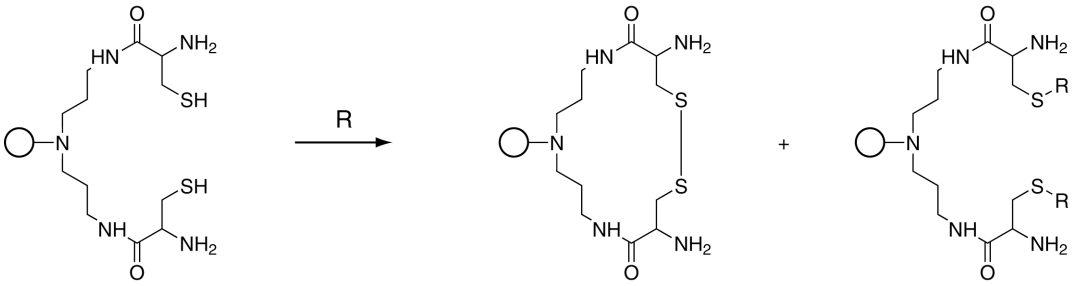


Figure 4.7 *ESI-MS spectra of **D3** under reducing conditions in a buffer at pH 2 and 0.1% of β-mercaptoethanol (left); under oxidizing conditions in a buffer at pH 8 and 0.1% of H_2O_2 (right).*

When dissolved in a buffer at neutral pH, an equilibrium was obtained for the interconversion between dithiol and disulfide in dendrimer **D1**. A heterogeneous mixture of dendrimers was obtained, some having no disulfide bridges, some having a few disulfide bridges and some dendrimers were fully oxidized. In order to perform native chemical ligation with these dendrimers, it was important that all cysteines were in the free thiol form. Therefore, native chemical ligation was carried out under reducing conditions (Chapter 3).
As analyzed using ESI-MS, oxidation and reduction for all thiol groups on dendrimers **D1** and **D2** was possible. Oxidation for all thiols on dendrimer **D3** also succeeded, but not all thiol groups could be reduced with BME and one disulfide bond persisted. This might explain the difficulties observed in characterization of **D3** modified with peptides (Chapter 3).

4.2.2 INTERMOLECULAR DISULFIDE BRIDGES

Besides the presence of intramolecular disulfide bridges, the occurrence of intermolecular disulfide bridges in the first, second and third generation cysteine-dendrimers was investigated. For that purpose, the dendrimers **D1-D3** were dissolved in water with β-mercaptoethanol (BME) as a model compound and hydrogen peroxide to close the (internal and external) sulfur bridges. Several conditions were studied: 0.1%, 1.0%, and 10% of BME, either with a low concentration (low: 0.4 eq relative to BME) or a high concentration (high: 16 eq relative to BME) of hydrogen peroxide relative to BME. By increasing the amount of BME, the amount of attached BME was expected to increase. An excess of peroxide would result in only even amounts of attached BME because complete oxidation of thiols will occur (Scheme 4.3).



Scheme 4.3 *Schematic representation of the reaction of a thiol-reactive probe (R) with a cysteine-dendrimer under oxidizing conditions, resulting in even amounts of probe attached to the dendrimer.*

As an example, in Figure 4.8 the ESI-MS spectrum was shown of **D3** with 1% of BME and a low amount of peroxide. In the *m/z* spectrum it was clearly seen that a mixture of products was obtained. The spectrum was much noisier compared to the spectrum of the dendrimer **D3** with no further additives (Figure 4.7). After deconvolution, a distribution was obtained ranging from no BME attached to 7 BME attached.

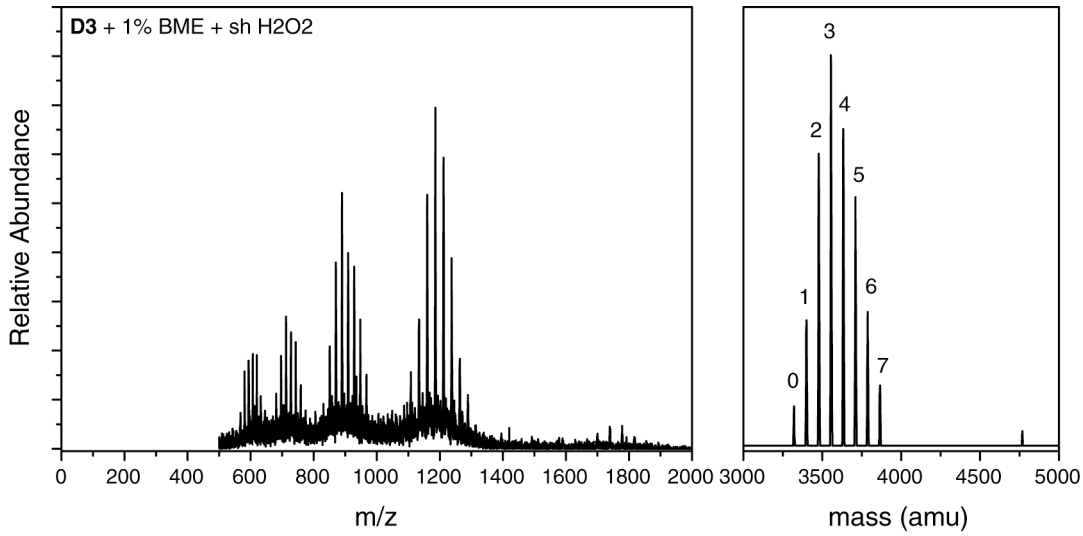


Figure 4.8 *ESI-MS spectrum and corresponding deconvoluted spectrum of **D3** with 1% of BME and low peroxide.*

A summary of the results obtained with the different generations of dendrimers was shown in Figure 4.9. As expected, when a higher concentrations of BME were used, more BME-groups were attached to the dendrimer. Even up to 16 (fully modified dendrimer **D3**) molecules of BME could be attached, suggesting that the fully BME-conjugated dendrimer was obtained. The use of high peroxide did not always result in full oxidation of the dendrimer. Especially in the case of **D3**, odd amounts of BME were still present. In only one case, full conversion to one product was seen; **D2** with 10% BME and high peroxide yielded the attachment of eight BME to the dendrimer as the only product.

Similar results were obtained for **D1** (four thiol groups) and **D2** (eight thiol groups). In the case of **D1**, up to four (fully modified dendrimer **D1**) could be attached and a preference for the attachment of even amounts of BME was seen. In the case of dendrimer **D2** up to eight

(fully modified dendrimer **D2**) could be attached and also here a preference for the attachment of even amounts of BME was seen.

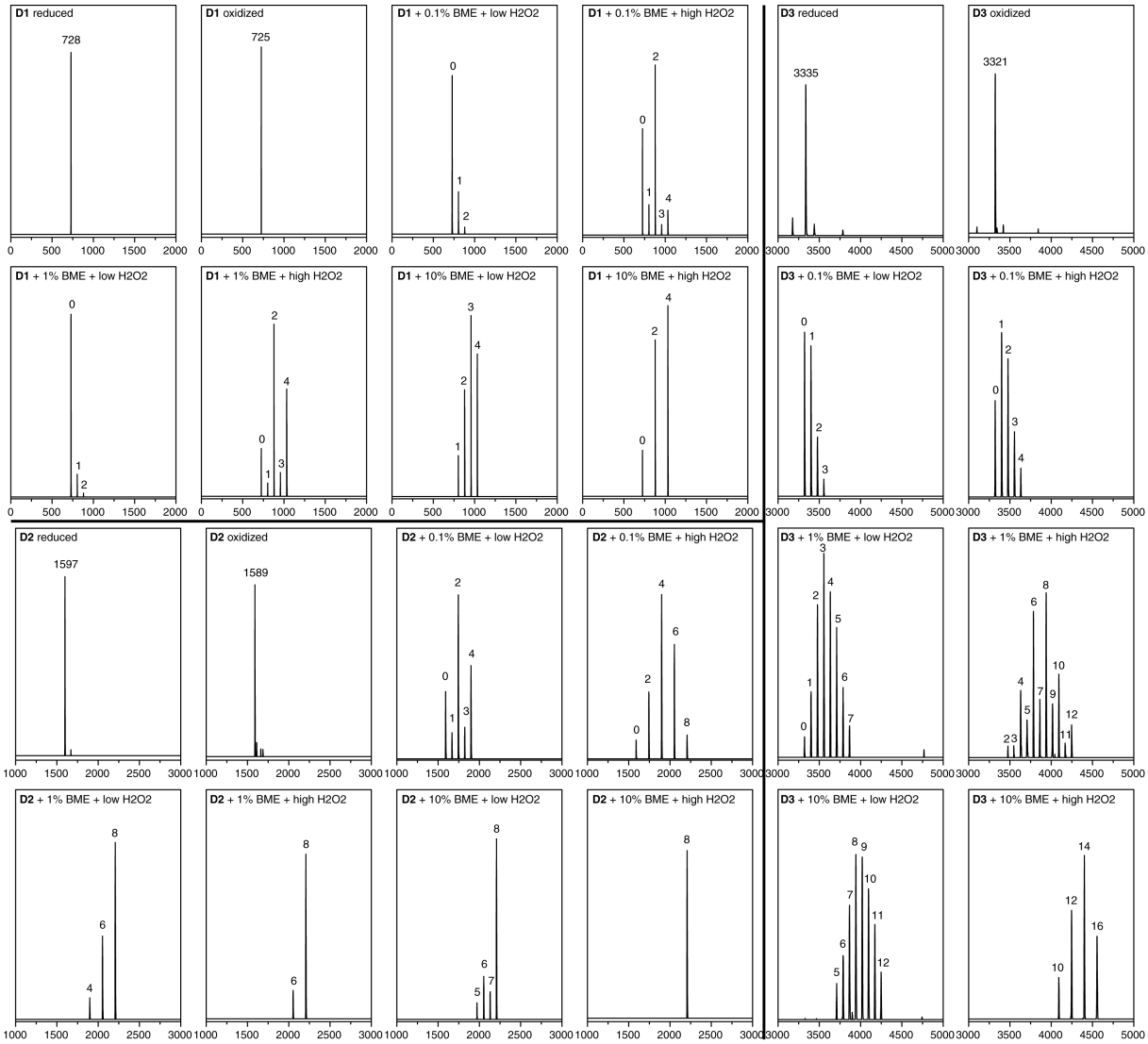


Figure 4.9 *Overview of deconvoluted mass spectra of **D1** (top left), **D2** (bottom left) and **D3** (right) with 0.1%, 1% and 10% of BME and low or high concentrations of hydrogen peroxide.*

In summary, intermolecular disulfide bridges could be formed by addition of BME and peroxide to the first, second and third generation cysteine dendrimers **D1**-**D3**. As expected, the amount of BME attached to the periphery of the dendrimer increased upon increasing concentration of BME. Furthermore, the use of a high concentration of peroxide resulted in a preferred attachment of even amounts of BME.

The experiments show that thiols on a cysteine dendrimer could be used for attachment of other thiol-containing compounds via intermolecular disulfide bridges. However, control over the attachment of exact amounts of BME could not be obtained. Therefore, in the next section, the use of a maleimide-thiol reaction for the chemical modification of peptide dendrimers will be discussed.

4.3. MALEIMIDE FUNCTIONALITIES

The pendant thiol groups at the cysteine dendrimer are not only reactive towards the formation of intra- or intermolecular disulfide bridges, but are also able to react with maleimides. The addition of probes having a maleimide functionality to the multivalent peptide dendrimers as described in Chapter 3 provides antoher tool for modification of these dendrimers. Not only can multiple peptides be attached to a single dendrimer, but in addition, probes can also efficiently be introduced in this way. For that reason, several maleimide functionalities (Figure 4.10) have been added to the first, second and third generation cysteine dendrimers **D1-D3** (section 4.3.1) that were used as a model system for peptide dendrimers. These syntheses were performed on an analytical scale. Maleimide **M1** itself and maleimide-β-alanine **M2** were used as models to study the maleimide reactivity. Maleimide-Oregon Green 488 **M4** was used for fluorescent labeling,[21] maleimide-biotin **M3** for biochemical labeling[26-28] and maleimide-LYRAG **M5** for the addition of multiple peptides to a single dendrimer scaffold. To develop biologically interesting molecules, the first generation peptide dendrimer with RGD (**D1P3**) was modified with maleimide-Oregon Green **M4** and maleimide-DTPA **M6** (Figure 4.15). These constructs were isolated and have potential use in imaging applications (section 4.3.2).

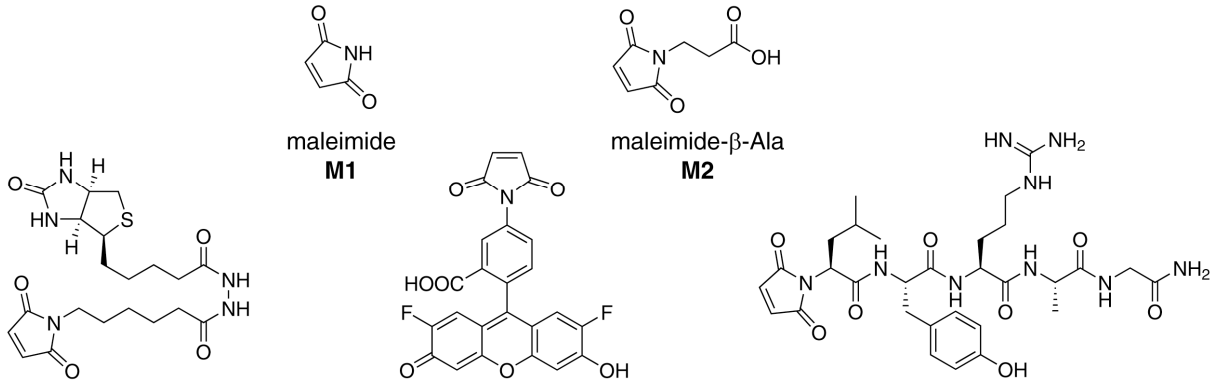
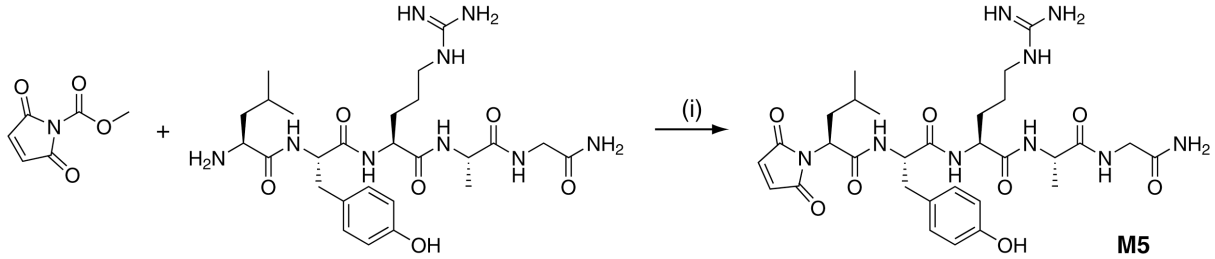


Figure 4.10 *Several maleimide functionalities: maleimide (**M1**); maleimide-β-alanine(**M2**); maleimide-biotin (**M3**); maleimide-oregon green 488 (**M4**); maleimide-Leu-Tyr-Arg-Ala-Gly (**M5**).*

4.3.1 REACTIONS WITH CYSTEINE DENDRIMERS

Maleimides **M1-M4** were commercially available, **M5** was synthesized using *t*Boc-mediated solid phase peptide synthesis (SPPS), cleaved from the resin with hydrogen fluoride and purified using preparative RP-HPLC. Methoxycarbonyl maleimide was added to LYRAG in a mixture of THF and a saturated solution of sodium bicarbonate in water. After one hour the product was purified using RP-HPLC combined with ESI-MS (Scheme 4.4).



Scheme 4.4 *Synthesis of mal-LYRAG from LYRAG and methoxycarbonyl-maleimide. Reaction conditions: (i) $NaHCO_3$ (aq, sat), THF.*

When introducing maleimide functionalities to cysteine dendrimers, a distribution of mainly even amounts of attached maleimides to dendrimers was once again expected; free thiols are reactive towards maleimides, whereas disulfide bridges are not (Scheme 4.3).
Solutions of **D1**, **D2**, and **D3** were made in water and a solution of maleimide was made in DMF. The maleimide solution was added to the dendrimer solution in such a way that the ratio of thiol and maleimide is 1:1 (to **D1** four equivalents of maleimide was added; to **D2** eight equivalents of maleimide was added; and to **D3** sixteen equivalents of dendrimer was added). The mixture was vigorously stirred for 5 minutes and subsequently analyzed using ESI-MS. A summary of deconvoluted data is represented in Figure 4.12.

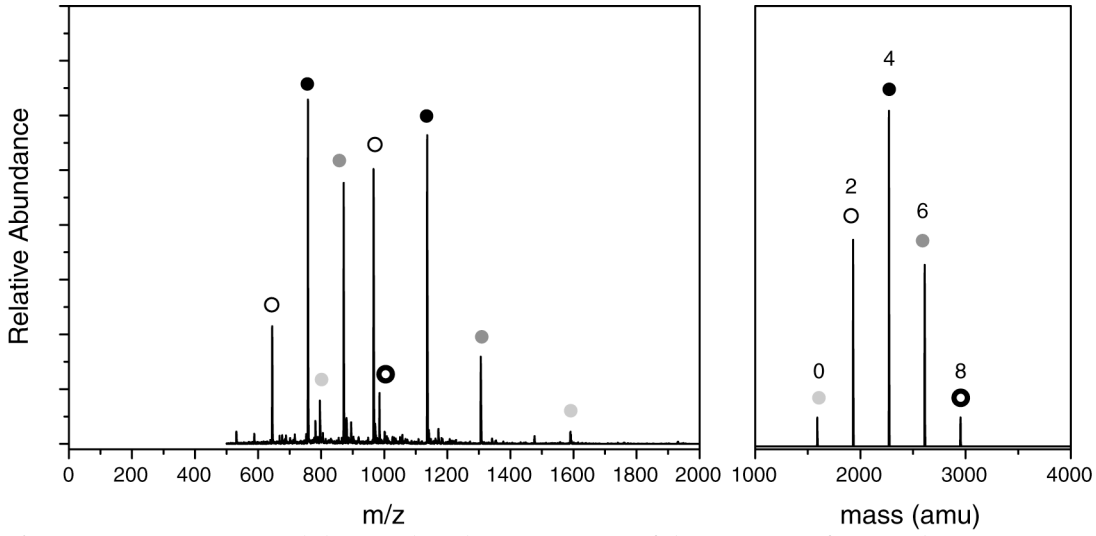


Figure 4.11 *ESI-MS and deconvoluted mass spectra of the reaction of **D2** with **M2**.*

As an example, the *m/z* and corresponding deconvoluted spectra of the reaction of **D2** with **M2** are shown (Figure 4.11). As expected, a distribution was found and only even amounts of maleimides were attached to the periphery of the dendrimer. Similar results were obtained for the reaction of **D1** or **D2** with **M1**-**M5**. The reaction of **M5** with **D3** also showed odd amounts of maleimides attached, indicating the reaction was not completed. For the reaction of **M3** and **M4** with **D3**, the obtained *m/z* spectra could not be deconvoluted.
Furthermore, it was observed that up to 16 peptides (**M5**) could be attached to the third generation dendrimer. This is of particular interest as with native chemical ligation, such products could not be analyzed (Chapter 3).

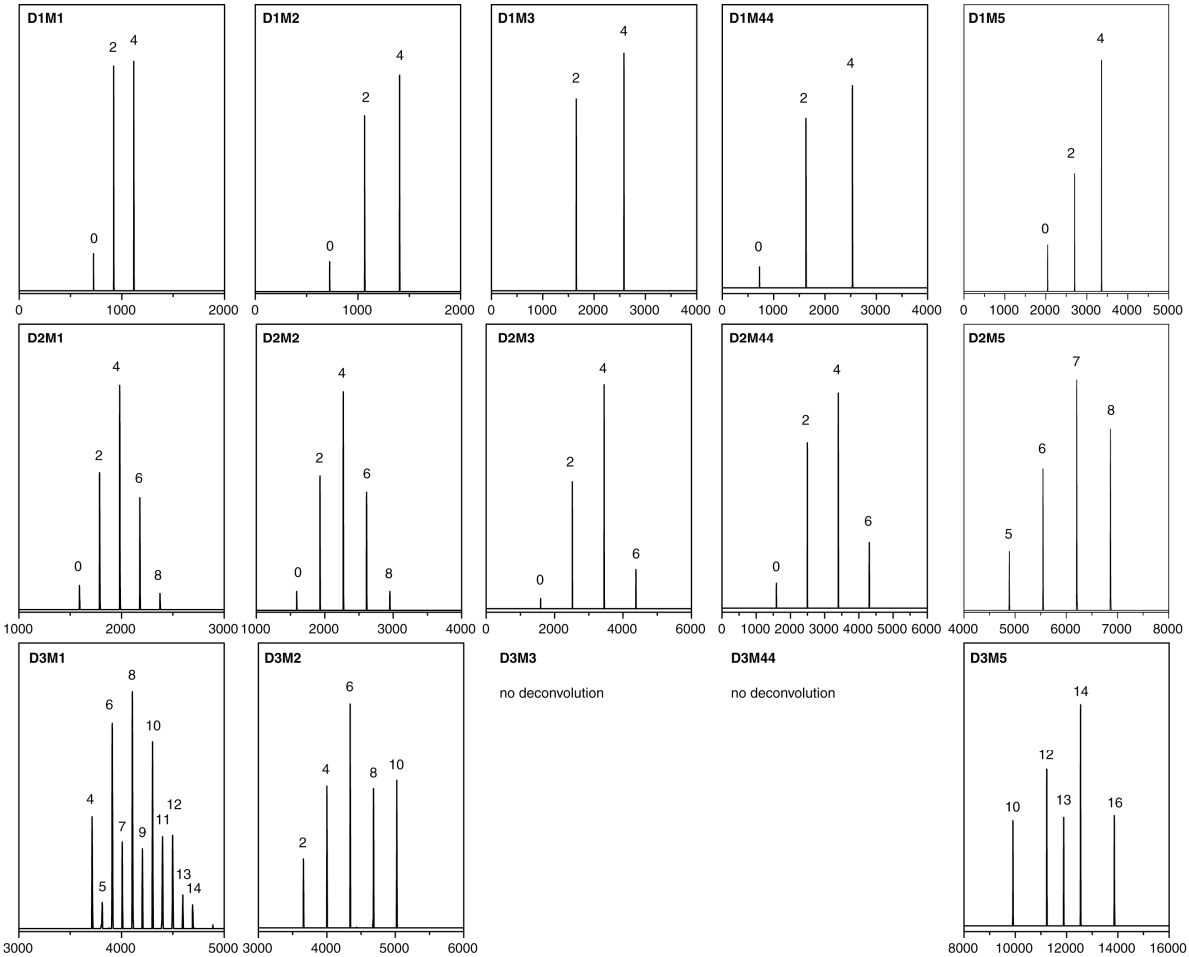


Figure 4.12 *Deconvoluted mass spectra of **D1**, **D2** and **D3** with **M1**, **M2**, **M3**, **M4** and **M5**.*

In all cases a distribution was found for the addition of even amounts of maleimides to the dendrimer scaffold. Only in case of reaction of **D3** odd amounts were observed in the mass spectra. Presumably, this is due to incomplete conversion of the reaction. Either one intramolecular disulfide bridge was formed or two maleimides were attached to the dendrimer. To obtain fully modified dendrimers, use of a reducing agent was necessary. The addition of BME as reducing agent is not possible as this compound is reactive towards maleimides, therefore TCEP (tris(2-carboxyethyl)phosphine hydrochloride, section 4.1) was used to reduce the disulfide bridges. The reactions with **D1**-**D3** and maleimide **M1** were carried out similar to the other maleimide reactions, only now 0.1% of TCEP was added during the reaction. After 1 hour of reaction time, the reaction went to completion and only the fully modified dendrimers were obtained. For future experiments, it is therefore recommended to perform maleimide reactions in the presence of TCEP to obtain full conversion of the thiols in the dendrimers.

It was shown that several maleimide probes were able to react with the first, second and third generation cysteine dendrimers. For the third generation even up to 16 peptides could be attached, a product that was difficult to analyze when native chemical ligation was used as coupling technique (Chapter 3). Although in all cases a distribution was found of added

maleimide probes, dendrimers could be fully functionalized by the addition of the reducing agent TCEP to the reaction; disulfide bridges were reduced and became reactive towards maleimides. As maleimide functionalization of cysteine dendrimers was successful, the modification of peptide dendrimers was studied next.

4.3.2 REACTIONS WITH PEPTIDE DENDRIMERS

It is of great interest for biological imaging applications to be able to functionalize peptide dendrimers with additional functionalities, *e.g.* other peptides, fluorescent probes or MRI probes. For this purpose, **D1P3** (Chapter 3) is used for modification since this dendrimer contains the target-specific sequence arginine-glycine-aspartic acid (RGD) that is known for binding to $\alpha_v\beta_3$ integrins expressed at the surface of cells involved in angiogenesis.[29-33]

To test the reactivity of the thiols of a peptide dendrimer, maleimide-LYRAG **M5** was coupled to **D1P3** on analytical scale. The reaction of **M5** with **D1P3** was performed similar to the maleimide experiments described earlier. The reaction mixture was analyzed using ESI-MS, and a distribution of products was obtained corresponding to 1, 2, 3, and 4 times **M5** attached to the dendrimer.

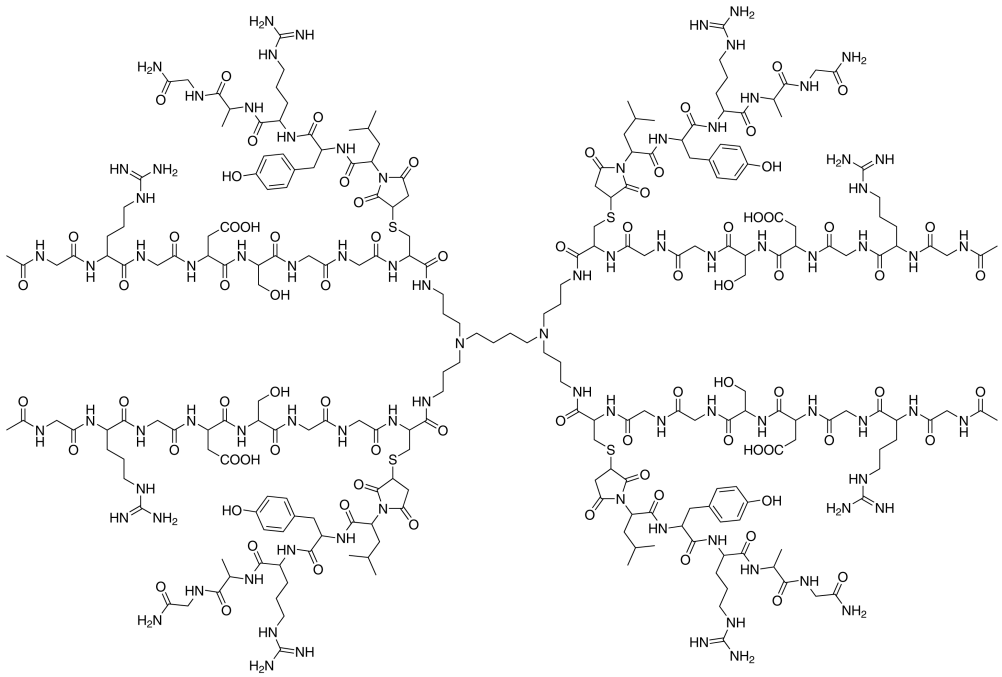


Figure 4.13 *Structural formula of **D1P3M5**; a multivalent scaffold was obtained containing four RGD-peptides as well four LYRAG-peptides.*

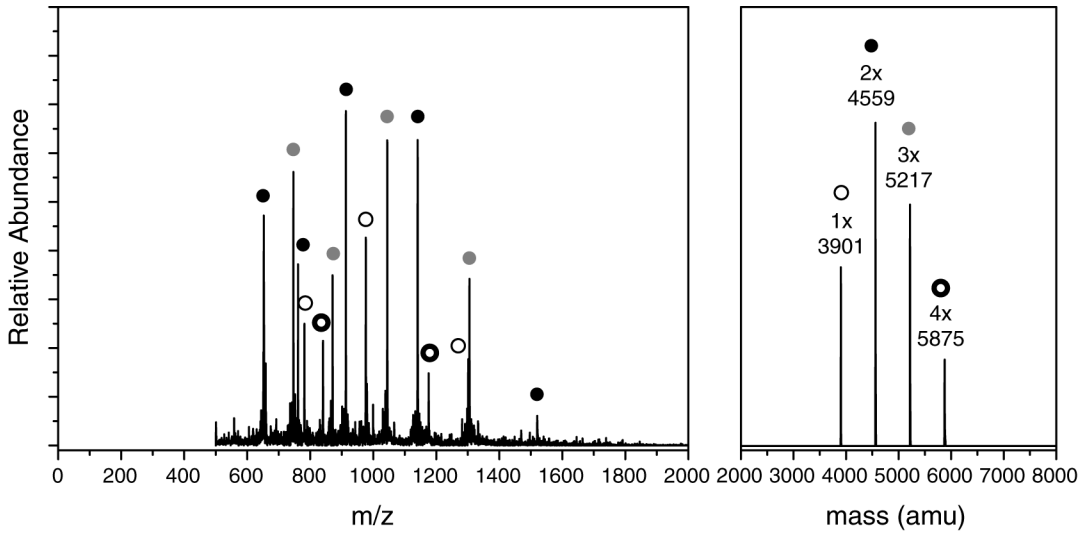


Figure 4.14 *ESI-MS and corresponding deconvoluted spectrum of **D1P3M5**; a mixture is obtained of one to four LYRAG-peptides attached.*

The mass spectra (Figure 4.14) showed that the reaction did not go to completion, likely explained by steric hindrance. Presumably, when using TCEP as reducing additive in this reaction, a well-defined structure is obtained, having four RGD peptides and four LYRAG peptides. Compared to the system described in Chapter 3, where a mixture is obtained, this is of great potential and can be regarded as a versatile tool for the chemical modification of peptide dendrimers. Therefore, in the next step, functional probes will be attached to peptide dendrimers on a preparative scale.

As it was shown on an analytical scale that the thiols of a peptide dendrimer are also reactive towards maleimides, preparative scale reactions were performed for the chemical modification of peptide dendrimers. A general strategy is reported for the introduction of multiple MRI labels and fluorescent labels for optical imaging to these scaffolds, utilizing highly efficient chemoselective reaction procedures and resulting in well-defined multivalent dendritic MRI contrast agents. The thioester-peptide AcGRGDSGG-MPAL **P3** was synthesized using *t*Boc-mediated solid phase peptide synthesis (Chapter 3). Native chemical ligation to the first generation cysteine dendrimer **D1** was performed and the product **D1P3** was obtained in 51% yield (Chapter 3).

The free thiol of the cysteine residue of dendrimer **D1P3** which took part in the ligation reaction was used to introduce a label, either a fluorescent label Oregon Green 488 (OG488) or a MRI label gadolinium(III) diethylenetriaminepentaacetic acid (Gd(III)DTPA). This was achieved through the reaction of the dendrimer **D1P3** with the maleimide of the corresponding label; maleimide-OG488 **M4** (commercially available) or maleimide-DTPA **M6**.[34]

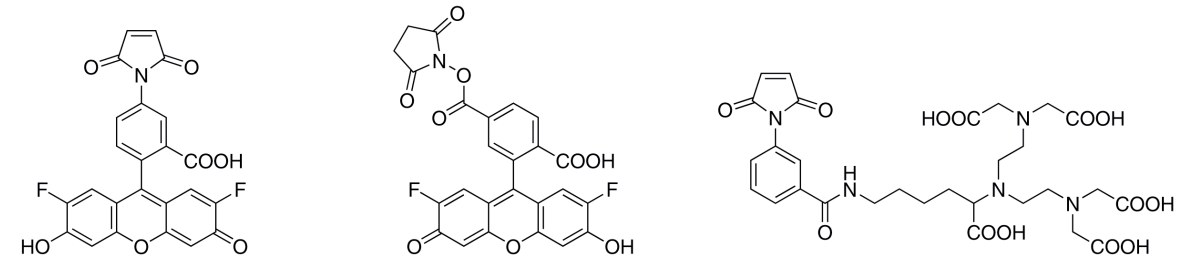


Figure 4.15 *Structural formula of maleimide-Orgeon Green 488* ***M4****, Oregon Green 488-succinimidyl ester* ***OG**** *and maleimide-DTPA* ***M6*** *(kindly provided by dr. A. Dirksen).*

Again, the reaction was monitored by analytical RP-HPLC and purified using preparative RP-HPLC, obtaining the labeled dendrimers **D1P3M4** in 22% yield and the DTPA functionalized dendrimer **D1P3M6** in 31% yield. The Gd(III) complex dendrimer **D1P3M6** could be made readily by addition of four equivalents of $GdCl_3$ in water yielding **D1P3M6-Gd** in a quantitative yield (>99%). All products were analyzed using ESI-MS (Table 4.1).

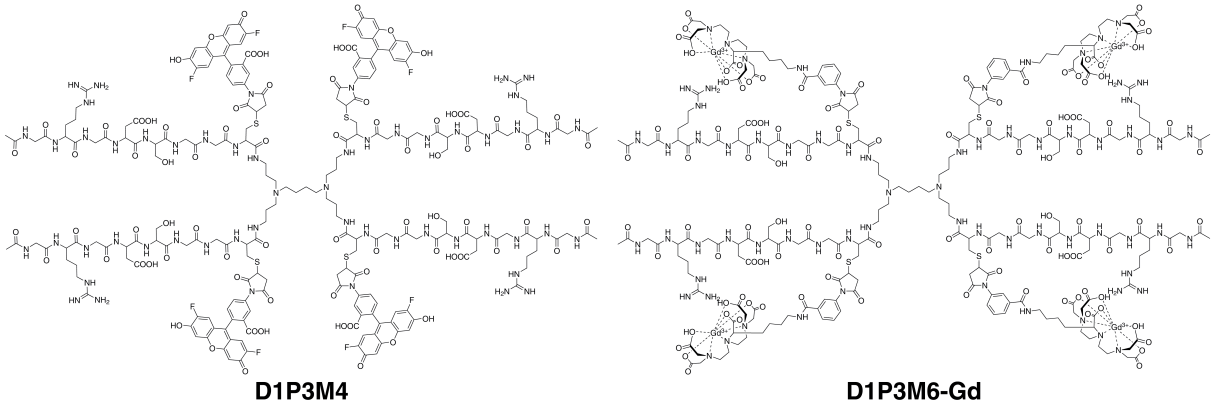


Figure 4.16 *Structural formula of* ***D1P3M4*** *(left) and* ***D1P3M6-Gd*** *(right).*

In addition, the esterification of amines on lysine residues for modification of peptide dendrimers was explored. To allow both imaging with MRI and optical methods of the same molecule, a bimodal multivalent peptide dendrimer was made. For this purpose, an extra lysine residue was incorporated in the peptide sequence, resulting in AcGRGDSGGKGG-MPAL **P10**. Adjusting the peptide sequence by introducing a lysine (K) residue after the RGD sequence is not expected to alter the specific binding of RGD, and offers an additional functional group that is reactive towards several activated esters. The thioester-peptide **P10** was synthesized using *t*Boc-mediated SPPS purified using RP-HPLC and analyzed using ESI-MS. The peptide was obtained in 61% yield.

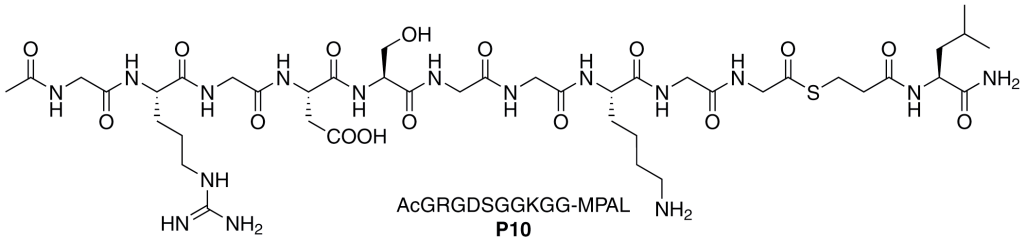


Figure 4.17 *Structural formula of AcGRGDSGGKGG-MPAL **P10**.*

The synthesis of **D1P10** was similar to the ligation reactions described earlier. Reaction of four equivalents of AcGRGDSGGKGG-thioester **P10** with **D1** under ligation conditions yielded the multivalent target specific peptide dendrimer **D1P10**. The reaction was monitored employing analytical RP-HPLC and after 1 hour the ligation product was purified using preparative RP-HPLC, obtaining the dendrimer in a yield of 46%.

The introduction of the fluorescent label Oregon Green 488 succinimidyl ester **OG*** (reactive towards the amine of lysine) and MRI label DTPA maleimide **M6** (reactive towards the thiol of cysteine) was done simultaneously on an analytical scale in sodium bicarbonate buffer. The reaction was monitored by analytical RP-HPLC and after 3 days the product was obtained. Also the product with two **M6** units attached was found. To reach full conversion, it is recommended to use TCEP as an additive in the reaction mixture.

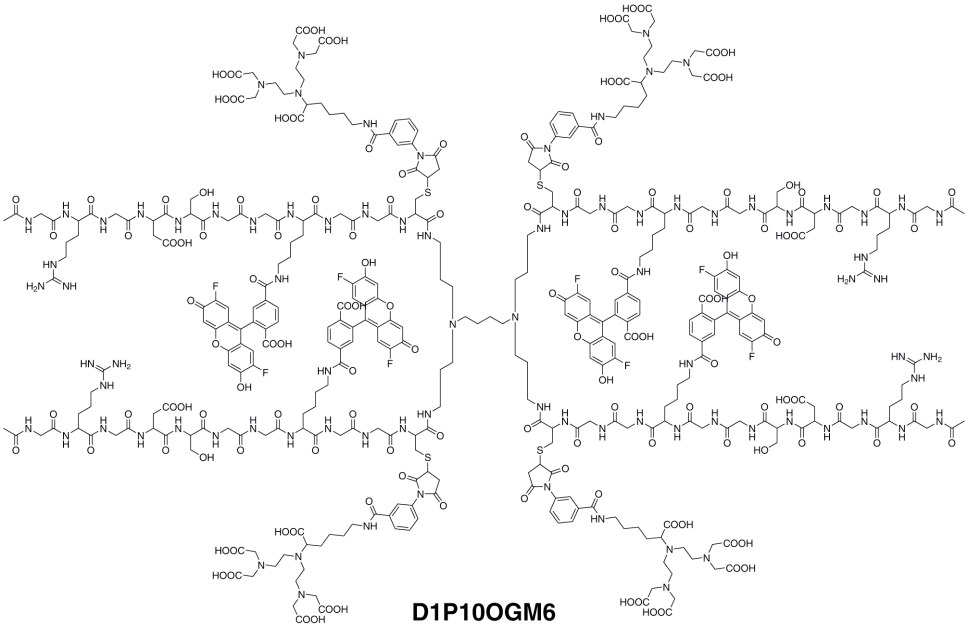


Figure 4.18 *Structural formula of **D1P10OGM6**.*

Table 4.1 *Observed and calculated mass of several peptide dendrimers.*

Peptide-dendrimer		Obs. mass (Da)	Calc. mass (Da)	Yield (%)
$[AcGRGDSGGC]_4$-dendrimer	**D1P3**	3243.2	3243.5	36-53% (27 mg)
$[AcGRGDSGGC(M4)]_4$-dendrimer	**D1P3M4**	5096.8	5096.9	22% (3 mg)
$[AcGRGDSGGC(M6)]_4$-dendrimer	**D1P3M6**	5897.8	5898.0	31% (6 mg)
$[AcGRGDSGGC(M6\text{-}Gd)]_4$-dendrimer	**D1P3M6-Gd**	6514.7	6514.9	>99%
AcGRGDSGGKGG-MPAL	**P10**	1088.7	1089.1	57% (42 mg)
$[AcGRGDSGGKGGC]_4$-dendrimer	**D1P10**	4212.4	4212.6	46% (22 mg)
$[AcGRGDSGGK(OG)GGC(M6)]_4$-dendrimer	**D1P10OGM6**	8444.1	8444.3	*n.i.**

*not isolated

The maleimide-thiol reaction as described above is a successful tool for introducing probes to peptide dendrimers. It was shown on preparative scale that imaging probes, such as fluorescent or MRI probes can be introduced on peptide dendrimers in good yields resulting in multivalent target specific imaging agents. Therefore, this strategy is a versatile synthetic tool for the chemical modification of peptide dendrimers. For drug delivery applications, it was investigated whether the maleimide-thiol reaction can be used to introduce dynamic covalent bonds at the periphery of a peptide dendrimer.

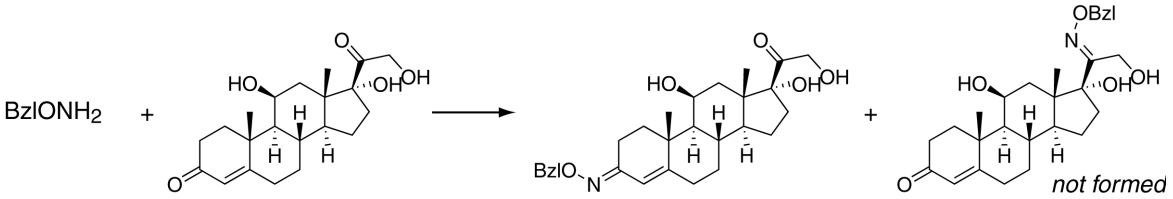
4.4. DRUG DELIVERY *VIA* REVERSIBLE OXIME FORMATION

Using the maleimide-thiol reaction it should also be possible to introduce dynamic covalent bonds, such as oximes. In this way a potential dynamic drug delivery system based on multivalent peptide dendrimers could be developed. As a proof of principle, the first generation cysteine dendrimer was used as a model system for chemical modification and cortisol as a drug model (Chapter 2). An oxime is formed by the reaction of an aminooxy group with an aldehyde or ketone, this bond is reversible depending on the pH of the environment.

4.4.1 CORTISOL REACTIVITY

Since cortisol has two ketone functionalities; one at the C-3 position and one at the C-20 position, it is relevant to know which ketone of cortisol is the most reactive with an aminooxy group, yielding the corresponding oxime. Therefore, some ^{1}H-NMR reference

experiments were carried out in methanol-*d4* with cortisol and *o*-benzylhydroxylamine as a model compound (Scheme 4.5).



Scheme 4.5 *Reaction of O-benzylhydroxylamine with cortisol. Reaction was performed in methanol. According to ^{1}H-NMR experiments the α,β-unsaturated ketone is the more reactive ketone of cortisol.*

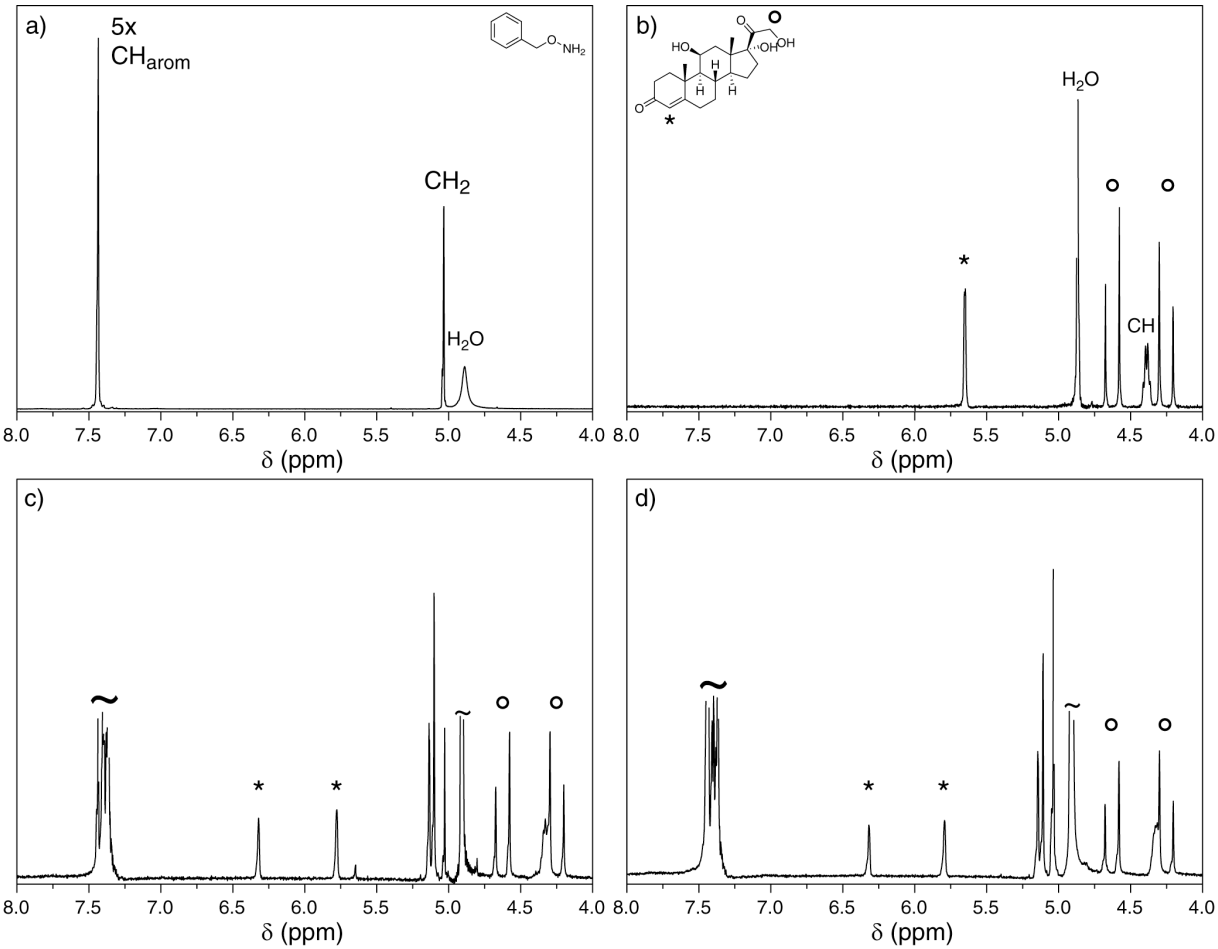


Figure 4.19 *^{1}H NMR in CD_3OD in the region of 8.0 ppm to 4.0 ppm of a) benzylhydroxylamine; b) hydrocortisone; c) benzylhydroxylamine and hydrocortisone in a ratio of 1:1; and d) in a ratio of 2:1 (excess benzylhydroxylamine).*

The ^{1}H-NMR of *o*-benzylhydroxylamine shows two singlets, one of the aromatic protons at 7.4 ppm and one at 5.0 ppm of the benzylic CH_2 protons. In the spectrum of cortisol, two peaks are of importance for this study, the singlet of the proton of CH of the α,β-unsaturated ketone (C-4, marked with a star in the spectrum) at 5.7 ppm and a double doublet at 4.25 ppm and 4.6 ppm corresponding to the CH_2 at the C-21 position (marked with two open circles in the spectrum). These peaks correspond to the protons that are next to the two

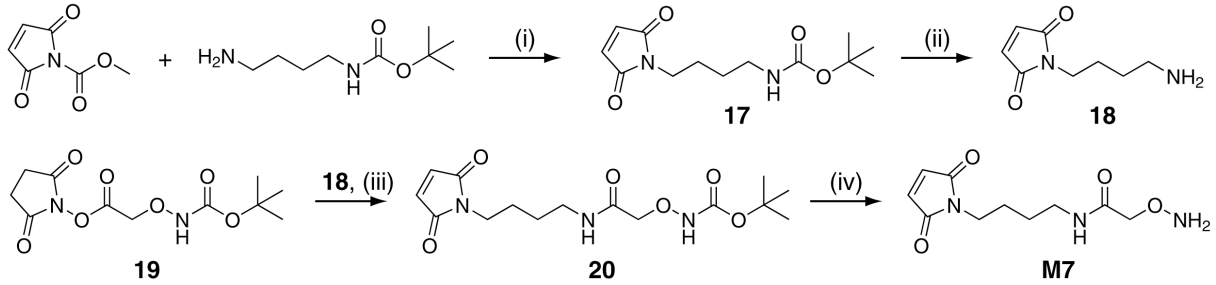
ketone functionalities of cortisol and will shift upon oxime formation. Also shown in the spectrum of cortisol is the proton of CH at C-11 as a quartet at 4.4 ppm. No preference for the *E* or *Z* isomer is expected to be found.

Two experiments were done: in the first experiment a 1:1 ratio of *o*-benzylhydroxylamine and cortisol is mixed in methanol-d_4, and in the second experiment a two-fold excess of *o*-benzylhydroxylamine was added. A clear shift and splitting of the CH signal of cortisol at C-4 was observed in the ^{1}H-NMR spectrum; from a singlet at 5.7 ppm it split into two singlets at 5.8 ppm and 6.3 ppm, whereas the double doublet of the CH_2 at C-21 remained unchanged. This indicated that the oxime formation took place only at the C-3 ketone, and not at the C-20 ketone. Even when an excess of *o*-benzylhydroxylamine was added, the only reactive ketone was the one at the C-3 position. Furthermore, the singlet of CH_2 of benzyl split into two singlets, indicating that the two isomers *E* and *Z* were formed.

As shown by ^{1}H-NMR spectroscopy, the α,β-unsaturated ketone of cortisol was the most reactive towards the formation of an oxime bond and both the *E* and *Z* isomers were found. To be used for the connection of cortisol to a cysteine-dendrimer, a coupling agent had to be synthesized consisting of both a maleimide function for attachment to the dendrimer and an aminooxy group for reaction with the cortisol-ketone.

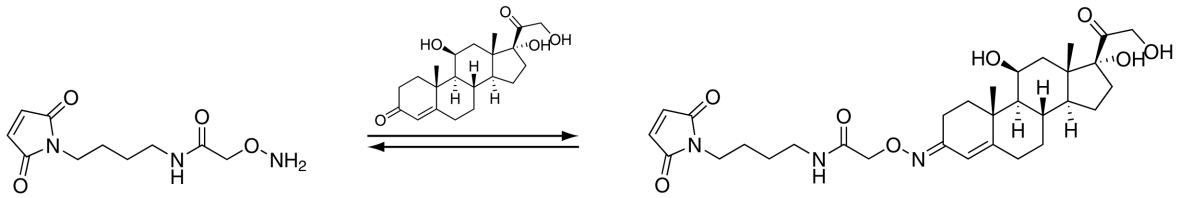
4.4.2 SYNTHESIS MALEIMIDE-AMINOOXY COUPLING AGENT

The maleimide-aminooxy coupling agent **M7** was synthesized from methyloxycarbonylmaleimide and mono-*tert*-butyloxycarbonylbutanediamine in 4 steps (Scheme 4.6). Methoxycarbonyl-maleimide was reacted with mono-*tert*-butyloxycarbonyl-protected butanediamine yielding *tert*-butyloxycarbonyl-protected maleimide compound **17**. After removal of the protective group by TFA, the TFA-salt of the maleimide-amine compound **18** was obtained in 70% yield. The acid functionality of 2-(*tert*-butoxycarbonylaminooxy) acetic acid was activated with hydroxysuccinimide in DMF with dicyclohexylcarbodiimide to form the succinimide ester **19**. Compound **19** was reacted with the TFA salt of the maleimide-amine compound **18** in dichloromethane in the presence of base yielding **20** in 77%. The *t*Boc-group was removed by TFA at 0 °C and the coupling agent **M7** was purified using RP-HPLC. The product was obtained in 46% yield as a yellow oil.



Scheme 4.6 *Synthesis of maleimide-aminooxy **M7**. Reaction conditions: (i) saturated solution of sodium bicarbonate in water, tetrahydrofuran; (ii) trifluoroacetic acid. (iii) Et_3N, dichloromethane; (iv) trifluoroacetic acid.*

To test the activity of **M7** towards oxime formation, an experiment was performed in buffered media in which **M7** was reacted with cortisol. The product was analyzed with RP-HPLC combined with ESI-MS and this indicated that the desired product was formed (found mass: 586.3 Da, calculated mass: 585.6 Da) (Scheme 4.7).



Scheme 4.7 *Reversible binding of cortisol to aminooxy functionality via oxime formation.*

Maleimide-aminooxy coupling agent **M7** was successfully synthesized and obtained in good yield. The aminooxy group of **M7** was reactive towards the C-3 ketone of cortisol and cortisol could be attached via an oxime bond.

4.4.3 DEVELOPMENT OF A DYNAMIC MULTIVALENT DRUG DELIVERY VEHICLE

Since it was demonstrated that **M7** was capable of binding cortisol, the step to dendrimers was made. Therefore, the first generation cysteine dendrimer **D1** was modified with four maleimide-aminooxy groups via the maleimide-thiol reaction (section 4.3). The synthesis was done in Tris buffer at pH 7.0 and the product was purified using RP-HPLC. The ESI-MS spectrum of the product **D1M7** showed a distribution of peaks (Figure 4.20). When zooming in to the area of interest (Figure 4.20 insert), a multiple deletion of 16 Da up to four times was shown, presumably corresponding to the loss of multiple $-NH_2$ groups.

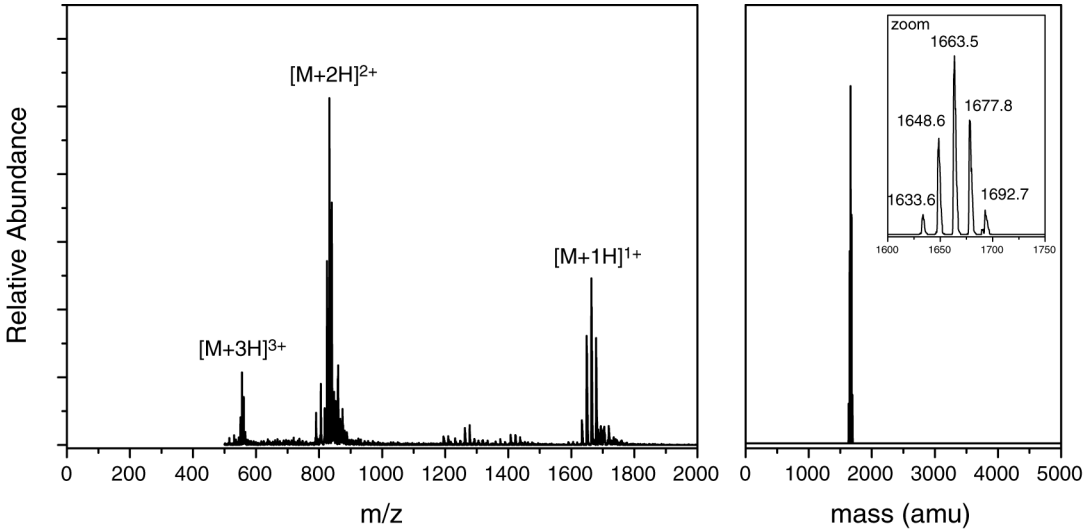


Figure 4.20 *ESI-MS spectrum and corresponding deconvoluted spectrum of **D1M7**.*

The addition of cortisol to **D1M7** was performed by dissolving **D1M7** in methanol and adding a solution of cortisol in methanol. The sample was analyzed with ESI-MS. A distribution was found ranging between 1 and 4 cortisol molecules attached to the dendrimer. The peak in the deconvoluted spectrum at 3072 Da corresponded to four molecules of cortisol attached (Figure 4.21). However, it should be mentioned that the peak

at 2711 Da corresponded to the dendrimer with three molecules of cortisol attached but lacking an $-NH_2$ group; the peak at 2352 Da had two molecules of cortisol and lacking two $-NH_2$ groups, and the peak at 1993 Da had only one cortisol and lacking three $-NH_2$ groups. This was in agreement with the finding that aminooxy dendrimers showed a distribution in the mass spectrum of the correct product peak and the peaks corresponding to the product lacking 1-4 $-NH_2$ groups. Therefore, it could be concluded that the aminooxy group was labile under the reaction conditions used. However, the results presented here indicated that cortisol-dendrimers could be formed on an analytical scale resulting in a dendritic drug delivery vehicle. Future research should focus on modifying the reactions conditions to ensure the stability of the aminooxy group.

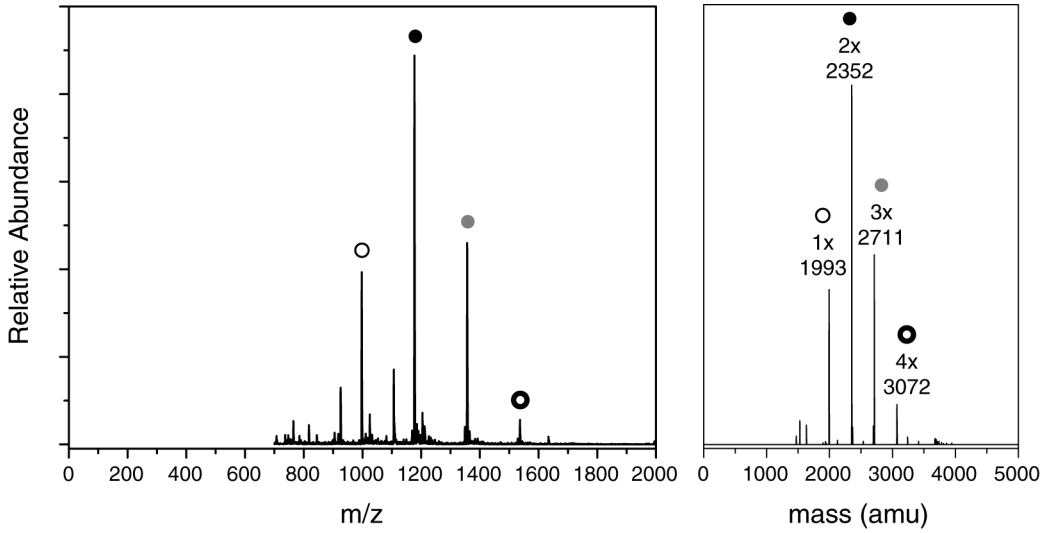


Figure 4.21 *ESI-MS spectrum and corresponding deconvoluted spectrum of **D1M7**-cortisol. It shows the distribution of 1-4 molecules of cortisol attached to the dendrimer.*

To introduce cortisol to cysteine dendrimers, a coupling agent was prepared having both a maleimide function for coupling to the dendrimer and an aminooxy group for oxime bond formation with cortisol. Using this coupling agent, a first generation cysteine dendrimer was successfully modified with aminooxy groups and cortisol could be attached. Although the reactions were performed with cysteine dendrimers as a model system, the results look promising for application to peptide dendrimers. Further optimization of reaction conditions is recommended to prevent dissociation of amino groups from the dendrimer. This could finally result in multivalent target specific drug delivery vehicles based on peptide dendrimer scaffolds.

4.5. CONCLUSIONS

The importance and use of thiols in the modification of well-defined peptide dendrimers was shown in this chapter. Thiols in cysteine dendrimers could be fully oxidized and reduced for the first and second generation. The thiols in the third generation were not fully reduced, which might explain the difficulties observed in the ligation of peptides to **D3** (Chapter 3). Thiol groups were also reactive towards BME and several maleimide probes could be attached to the first, second and third generation cysteine dendrimers **D1-D3**. In all cases a distribution of products was obtained according to ESI-MS and a preference for the attachment of even numbers of maleimides was observed. To obtain only the fully modified dendrimer TCEP was added, which is recommended for future experiments. For the addition of a maleimide peptide **M5** to **D3**, even up to 16 peptides attached to the dendrimer could be achieved, this product was difficult to analyze when native chemical ligation was employed as the coupling technique (Chapter 3). In addition, well-defined first generation peptide dendrimers with two different peptides were made. A multivalent target-specific dendritic MRI contrast agent was synthesized on preparative scale using the combination of native chemical ligation and the maleimide-thiol reaction. Moreover, oxime chemistry was used in combination with maleimide-thiol reaction for the modification of peptide dendrimers to yield a potential dynamic drug delivery vehicle. The methodology presented in this chapter can be considered a general approach for the modification of peptide dendrimers resulting in target-specific dendritic MRI contrast agents.

4.6. EXPERIMENTAL SECTION

General methods

Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification. 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *t*Boc-amino acids were obtained from NovaBiochem (USA). Trifluoroacetic acid (TFA) was obtained from Halocarbon (USA). N,N-diisopropylethylamine (D^iPEA) was obtained from Applied Biosystems (USA). Oregon Green succinimidyl ester was obtained from Molecular Probes Inc (USA). Peptide synthesis grade N,N-dimethylformamide (DMF) and HPLC grade acetonitrile (CH_3CN) were purchased from Biosolve. HF was purchased from Matheson Gas (USA). Water was demineralized prior to use. Analytical RP HPLC was performed on a Varian Prostar HPLC system with a Vydac™ protein peptide C18 column (0.5 × 15 cm, flow 1 mL/min), eluted with a linear gradient of 0–60% CH_3CN in H_2O (+0.1% TFA) in 30 minutes. The HPLC system was coupled to an UV/vis detector probing at 214 nm. Semi-preparative HPLC was performed using a Vydac C18 column (2.5 × 20 cm, 10 mL/min), eluted with a linear gradient of 0–20% CH_3CN in H_2O (+0.1% TFA) in 90 minutes. LC-ESI-MS measurements were performed on a Finnigan LCQ Deca XP Max ion trap mass spectrometer (Thermo Electron Corporation, San Jose, USA). Deconvolution of the mass-to-charge ESI-MS spectra was performed with MagTran 1.02 software.[35]

*Oxidation-reduction dendrimer **D1-D3***

A solution of 10 mg/mL of dendrimer **D1-D3** was made in water. To 100 µL of this solution, 1 µL of β-mercaptoethanol (reducing conditions) or 1 µL of hydrogen peroxide (oxidizing conditions) was added. The samples were diluted 10 times before measuring the samples using ESI-MS.

*Addition of β-mercaptoethanol to **D1-D3***

A solution of 10 mg/mL of dendrimer **D1-D3** was made in water. To 100 µL of these solutions 0.1, 1, or 10 µL of β-mercaptoethanol was added. Respectively 0.02, 0.2 or 2 µL of hydrogen peroxide (shortage hydrogen peroxide) was added and the mixtures were vigorously stirred. The samples were diluted 10 times before measuring the samples using ESI-MS. Subsequently 0.7, 7 or 70 µL of hydrogen peroxide (excess hydrogen peroxide) was added to the reaction mixture and the samples were vigorously stirred. The samples were diluted 10 times before measuring the samples using ESI-MS.

*Addition of **M1** to **D1-D3***

To 100 µL of a 10 mg/mL-solution of **D1-D3** in water, respectively 11 (4.1 eq), 10 (8.2 eq), or 10 µL (17 eq) of a 50 mg/mL-solution of maleimide **M1** in DMF was added. The mixture was vigorously stirred for 15 minutes and measured using ESI-MS.

*Addition of **M2** to **D1-D3***

To 100 µL of a 10 mg/mL-solution of **D1-D3** in water, respectively 20 (4.3 eq), 17 (8.0 eq), or 17 µL (16.7 eq) of a 50 mg/mL-solution of maleimide-β-alanine **M2** in DMF was added. The mixture was vigorously stirred for 15 minutes and measured using ESI-MS.

*Addition of **M3** to **D1-D3***

To 100 µL of a 10 mg/mL-solution of **D1-D3** in water, respectively 50 (4.0 eq), 46 (8.1 eq), or 45 µL (16.6 eq) of a 50 mg/mL-solution of maleimide-biotin **M3** in DMSO was added. The mixture was vigorously stirred for 15 minutes and measured using ESI-MS.

*Addition of **M4** to **D1-D3***

To 100 µL of a 10 mg/mL-solution of **D1-D3** in water, respectively 51 (4.0 eq), 47 (8.1 eq), or 45 µL (16.2 eq) of a 50 mg/mL-solution of maleimide-oregon green 488 **M4** in DMF was added. The mixture was vigorously stirred for 15 minutes and measured using ESI-MS.

*Maleimide-LYRAG-NH_2 (**M5**)*

LYRAG was synthesized using *t*Boc-mediated SPPS and cleaved from the resin with HF. The crude product (162 mg, 0.28 mmol, 1 eq) was dissolved in 20 mL of a mixture of THF and a saturated

solution of sodium bicarbonate in water. Methoxycarbonylmaleimide was added (52 mg, 0.33 mmol, 1.2 eq) and after 4 hours the product was purified using RP-HPLC and obtained in 2% yield (5 mg).

*Addition of **M5** to **D1-D3***
To 12, 15, or 16 µL of a 10 mg/mL-solution of **D1-D3** in water, respectively 50 (4.0 eq), 50 (8.0 eq), or 50 µL (16.0 eq) of a 10 mg/mL-solution of maleimide-LYRAG **M5** in water was added. The mixture was vigorously stirred for 15 minutes and measured using ESI-MS.

*Diethylenetriaminepentaacetic acid (DTPA) maleimide **M6***
Maleimide-DTPA was kindly provided by dr. A. Dirksen.

*AcGRGDSGG-MPAL **P3***
See Chapter 3.

*AcGRGDSGGKGG-MPAL **P10***
After the synthesis of MPAL thioester to the resin (using *t*Boc-mediated solid phase peptide synthesis,[36] see Chapter 3 for details), subsequently, Boc-Gly, Boc-Gly, Boc-Lys, Boc-Gly, Boc-Gly, Boc-Ser(Bzl), Boc-Asp(O*c*Hxl), and Boc-Gly were coupled for 10 minutes each; Boc-Arg(Tos) was coupled for 20 minutes, and Boc-Gly was coupled for 10 minutes. The resin was washed and dried and the peptide sequence was cleaved from the resin using the HF cleavage procedure. The peptide was purified using RP-HPLC using a gradient of 5-25% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 61% yield (41.8 mg). ESI-MS: found deconvoluted mass: 1088.7 Da; calculated mass: 1089.1 Da.

*$[AcGRGDSGGC]_4$-dendrimer **D1P3***
See Chapter 3.

*$[AcGRGDSGGC(OG)]_4$-dendrimer **D1P3M4***
Dendrimer **D1P3** (8.7 mg, 2.6 µmol, eq) was dissolved in 200 µL 0.1 M Tris (aq, pH 7.0). The pH of the solution was measured (7.0) and the solution was added to maleimide-oregon green 488 **M4** (5 mg, 10.7 µmol, 4 eq). The reaction mixture continued for 1 h at room temperature. The product was purified employing semi-preparative RP HPLC over a C18 column with a gradient of 9–27% CH_3CN in H_2O (+0.1% TFA) in 90 minutes. Freeze drying yielded **D1P3M4** (3 mg, 0.6 µmol, 22%) as a fluffy white powder. ESI-MS: found deconvoluted mass: 5096.8 Da; calculated mass: 5096.9 Da.

*$[AcGRGDSGGC(DTPA)]_4$-dendrimer **D1P3M6***
Dendrimer **D1P3** (11 mg, 3.4 µmol) was dissolved in 500 µL 0.1 M Tris (aq, pH 6.9). The pH of the solution was adjusted to pH 6.5 by the addition of small aliquots of 1.0 M NaOH (aq) and subsequently added to maleimide-functionalized DTPA (9 mg, 0.13 µmol). The reaction mixture continued for 1 h at room temperature at pH 6.5. The reaction was monitored employing analytical RP HPLC over a C18 column with a gradient of 0–60% CH_3CN in H_2O (+0.1% TFA) in 30 minutes. Analytical RP HPLC: product eluting at 11.2 min. The reaction mixture was filtered and the product was purified employing semi-preparative RP HPLC over a C18 column with a gradient of 9–27% CH_3CN in H_2O (+0.1% TFA) in 90 minutes. Freeze drying yielded **D1P3M6** (6.3 mg, 1.1 µmol, 31%) as a fluffy white powder. ESI-MS: found deconvoluted mass: 5897.9 Da; calculated: 5897.8 Da.

*$[AcGRGDSGGC(DTPA)]_4$-dendrimer **D1P3M6-Gd***
The dendrimer **D1P3M6** (1 mg, 0.17 µmol) was dissolved in water (0.1 mL). The pH of the aqueous solution was adjusted to 6.5–7.0 by adding small aliquots of 0.25% NH_4OH (aq). Subsequently, a solution of $GdCl_3 \cdot 6\ H_2O$ (0.25 mg, .68 µmol) in water (0.1 mL) was added while maintaining the pH at 7 with a 0.25% NH_4OH (aq) solution. The solution was stirred vigorously for 2 h at room temperature. The formation of the complex was confirmed with LC-ESI-MS (ProSphere column). After freeze drying the product **D1P3M6-Gd** was obtained as a white powder in quantitative yield. ESI-MS: found deconvoluted mass: 6514.7 Da; calculated mass: 6514.9 Da.

*$[AcGRGDSGGKGGC]_4$-dendrimer **D1P10***
AcGRGDSGGKGG-MPAL **P10** (50 mg, 46 µmol) and the first generation of a cysteine-functionalized poly(propylene imine) dendrimer **D1** (8.4 mg, 11 µmol) were dissolved in 1 mL of 6 M guanidine, 0.07

M Tris (aq) pH 8. To this solution 25 µL (2 v-%) of thiophenol and 25 µL (2 v-%) of benzylmercaptan were added. The pH was adjusted to pH 7.5 by the addition of small aliquots of 1.0 M NaOH (aq). The reaction was continued for 60 min at 37 ˚C. The reaction mixture was filtered and the product was purified employing semi-preparative RP-HPLC over a C18 column with a gradient of 5–25% CH_3CN in H_2O (+0.1% TFA) in 90 minutes. Freeze drying yielded **D1P10** (22.6 mg, 5.3 µmol, 46%) as a fluffy white powder. ESI-MS: found deconvoluted mass: 4212.4 Da; calculated mass: 4212.6 Da.

[AcGRGDSGGK(OG488)GGC(DTPA)]$_4$-dendrimer ***D1P10OGM6***
The reaction was done on analytical scale. Dendrimer **D1P10** (4.3 mg, 1.0 µmol, 1 eq) was dissolved in 400 µL sodium bicarbonate buffer (pH 8.0). The solution was added to **M6** (3.1 mg, 4.6 µmol, 4.6 eq) and the pH was adjusted to pH 7.0 by the addition of small aliquots of 1.0 M NaOH (aq). The reaction was performed at room temperature and monitored by RP-HPLC. After 1.5 hour the reaction was completed and Oregon green 488 succinmidyl ester **OG*** (2.7 mg, 5.3 mmol, 5.3 eq) was added. The reaction was monitored by RP-HPLC and after 3 days, the reaction was completed.

NMR experiments o-benzylhydroxylamine and cortisol
Equimolar amounts of cortisol and o-benzylhydroxylamine: cortisol (10 mg, 28 µmol, 1 eq) dissolved in 500 µL of methanol-*d4* was added to a solution of o-benzylhydroxylamine (4.4 mg, 28 µmol, 1 eq) in 500 µL of methanol-*d4*. The solution was vigorously stirred and after 30 minutes the sample was measured with ^{1}H-NMR. Similar conditions were used for the NMR experiment in which 2 equivalents of o-benzylhydroxylamine (8.8 mg, 55 µmol, 2 eq) was used.

tert-*Butyl 4-aminobutylcarbamate*
This compound was kindly provided by the Junior Researches Institute.
^{1}H-NMR ($CDCl_3$) δ (ppm): 4.8 (s, 1H, NH), 3.65 (s, 2H, NH_2), 3.05 (q, 2H, CH_2), 2.65 (t, 2H, CH_2), 1.45 (m, 4H, 2×CH_2), 1.4 (s, 9H, 3×CH_3). ^{13}C-NMR ($CDCl_3$) δ (ppm): 155.9 (C_q, CO), 78.7 (C_q, *t*Boc), 66.9 (dioxane), 41.7 (CH_2NH_2), 40.3 (CH_2NH), 30.8 ($CH_2CH_2NH_2$), 28.3 (3×CH_3), 27.3 (CH_2CH_2NH).

tert-*Butyl 4-(2,5-dioxo-2H-pyrrol-1(5H)-yl)butylcarbamate* ***17***
A saturated solution of sodium bicarbonate in water was mixed with THF. Mono-*t*Boc-protected butanediamine (1.6 g, 8.6 mmol, 1 eq) was dissolved in 20 mL of this mixture and the reaction was cooled to 0 °C. methoxycarbonylmaleimide (1.59 g, 10.2 mmol, 1.2 eq) was added in small portions and the reaction was stirred for 4 hours at room temperature. The pH was checked regularly and extra solvent was added when precipitates were seen. The product was extracted 3 times with ethyl acetate. The organic layers were combined, washed with brine and dried over magnesium sulfate. The solvent was evaporated *in vacuo* and the product was obtained in 86% yield (1.9 g). ^{1}H-NMR ($CDCl_3$) δ (ppm): 6.7 (s, 2H, CH), 4.6 (s, 1H, NH), 3.4 (t, 2H, CH_2N), 3.15 (q, 2H, CH_2NH), 1.6 (p, 2H, CH_2), 1.45 (m, 2H, CH_2), 1.4 (s, 9H, 3×CH_3).

1-(4-Aminobutyl)-1H-pyrrole-2,5-dione ***18***
Compound **17** was cooled to 0 °C and TFA was slowly added. After stirring for 1 hour at 0°C, TFA was evaporated *in vacuo* and the product was precipitated from diethyl ether and obtained as the TFA salt in 70% yield (0.73 g). ^{1}H-NMR (DMSO-*d6*) δ (ppm): 7.0 (s, 2H, CH), 3.5 (t, 2H, CH_2N), 2.8 (q, 2H, CH_2NH), 1.25 (m, 4H, 2×CH_2).

*2,5-Dioxopyrrolidin-1-yl 2-(*tert-*butoxycarbonylaminooxy)acetate* ***19***
2-(*tert*-butoxycarbonylaminooxy)acetic acid (2.0 g, 10.5 mmol, 1 eq) was dissolved in 10 mL of DMF. The reaction mixture was cooled to 0 °C and performed under an argon flow. Respectively *N*-hydroxysuccinimide (1.2 g, 10.5 mmol, 1eq) and *N*,*N*-dicyclohexylcarbodiimide (DCC, 2.16 g, 10.5 mmol, 1 eq) were added and the reaction was stirred overnight. The suspension was filtered over a glass filter and DMF was evaporated. The product was recrystallized from isopropanol and obtained as white powder in 80% yield (1.1 g). ^{1}H-NMR ($CDCl_3$) δ (ppm): 7.8 (s, 1H, NH), 4.8 (s, 2H, CH_2), 2.9 (s, 4H, 2×CH_2), 1.5 (s, 9H, 3×CH_3). ^{13}C-NMR ($CDCl_3$) δ (ppm): 168.3 (2×CO, Suc), 164.7 (CO, ester), 155.9 (CO, *t*Boc), 82.3 (C_q, *t*Boc), 70.5 (CH_2), 27.7 (3×CH_3), 25.2 (2×CH_2, Suc).

tert-*Butyl 2-(4-(2,5-dioxo-2H-pyrrol-1(5H)-yl)butylamino)-2-oxoethoxycarbamate* ***20***
Compound **18** (0.5 g, 1.77 mmol, 1 eq) was dissolved in 5 mL of dichloromethane and triethylamine (0.37 mL, 2.66 mmol, 1.5 eq) was added. Compound **19** (0.51, 1.77 mmol, 1 eq) was added and the mixture was stirred overnight. The reaction mixture was subsequently washed with a saturated solution of potassium bisulfite, a saturated solution of potassium bicarbonate and brine. The organic layers were combined, dried over magnesium sulfate and the solvent was evaporated *in vacuo*. The product was obtained in 77% yield (0.46 g) as yellow oil. ^{1}H-NMR ($CDCl_3$) δ (ppm): 8.2 (s, 1H, NH), 7.9 (s, 1H, NH), 6.7 (s, 2H, CH), 4.3 (s, 2H, CH_2), 3.5 (t, 2H, CH_2), 3.3 (q, 2H, CH_2), 1.6 (m, 4H, 2×CH_2), 1.4 (s, 9H, 3×CH_3).

2-(Aminooxy)-N-(4-(2,5-dioxo-2H-pyrrol-1(5H)-yl)butyl)acetamide ***M7***
Compound **20** (0.46 g, 1.3 mmol) was cooled to 0 °C and TFA was slowly added. After stirring for 1 hour at 0°C, TFA was evaporated *in vacuo* and the product was precipitated from diethyl ether. The crude product was obtained as the TFA salt in 89% yield (0.42 g). The product (33.6 mg) was purified using RP-HPLC using a gradient of 15-35% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. **M7** was obtained in 51% yield (17.2 mg). ^{1}H-NMR (CD_3OD) δ (ppm): 6.8 (s, 2H, CH), 4.6 (s, 2H, CH_2), 3.5 (t, 2H, CH_2), 3.2 (q, 2H, CH_2), 1.5 (m, 4H, 2×CH_2).

M7 *+ cortisol*
The reaction was performed on analytical scale. 500 µL of a 10 mg/mL-solution of **M7** in methanol was added to 510 µL of a 10 mg/mL-solution of cortisol in methanol. The mixture was vigorously stirred, a sample was taken and diluted 5 times with water and measured with ESI-MS. ESI-MS: found mass (*m/z*): 586.3 Da; calculated mass: 585.6 Da.

[Aminooxy]$_4$-dendrimer ***D1M7***
The first generation cysteine-dendrimer **D1** (11.1 mg, 15 µmol, 1 eq) was dissolved in 1 mL 0.1 M Tris buffer of pH 7.0. **M7** (15 mg, 62 µmol, 4.1 eq) was added and the reaction was stirred for 1 hour at room temperature. The product was purified using RP-HPLC using a gradient of 5-25% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure product was obtained in 24% yield (6.2 mg). ESI-MS: found deconvoluted masses: 1692.7 Da (M), 1677.8 Da (M-NH_2), 1663.5 Da (M-2NH_2), 1648.6 Da (M-3NH_2), 1633.6 Da (M-4NH_2); calculated masses: 1694.0 Da (M), 1679.0 Da (M-NH_2), 1664.0 Da (M-2NH_2), 1649.0 Da (M-3NH_2), 1634.0 Da (M-4NH_2).

[Cortisol-aminooxy]$_4$-dendrimer ***D1M7-cortisol***
The reaction was done on analytical scale. Cortisol (4.2g, 11 µmol, 4 eq) was dissolved in methanol and added to **D1M7** (5 mg, 2.9 mmol, 1 eq). The mixture was vigorously stirred and measured with liquid chromatography combined with mass spectrometry (LC-MS). ESI-MS: found decovoluted masses: 3072.3 Da (M), 2711.4 Da (M-cortisol-NH_2), 2352.3 Da (M-2cortisol-2NH_2), 1993.2 Da (M-3cortisol-3NH_2); calculated masses: 3071.9 Da (M), 2712.4 Da (M-cortisol-NH_2), 2352.9 Da (M-2cortisol-2NH_2), 1993.5 Da (M-3cortisol-3NH_2).

4.7. REFERENCES

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CHAPTER 5

PEPTIDE DENDRIMERS FOR BIOMEDICAL APPLICATIONS

ABSTRACT

Peptide dendrimers are promising drug delivery systems that can target specific tissues or organs. In this chapter, multivalent binding effects of peptide dendrimers to receptors are evaluated in vitro *and* in vivo *in a preliminary study and some specific examples of biomedical applications of such dendrimers are described. Peptide dendrimers are synthesized using native chemical ligation as described in Chapter 3. In the quest for a new synthetic vaccine against prostate cancer, derivatives of the gonadotropin-releasing hormone (GnRH) are coupled to the periphery of a dendrimer and tested for immune response in rats. It is tested whether the dendrimer scaffold can serve as a replacement for the keyhole limpet hemocyanin (KLH) carrier protein which is normally used in vaccinations. However, low immune responses are found. To investigate whether the low immune responses are due to the peptide dendrimer structure or the peptide sequence a second* in vivo *system is evaluated. Peptide dendrimers are synthesized containing part of the parasite circumsporozoite (CS) protein attached to the periphery and immune responses in rabbits are determined. When coupled to KLH, the peptide dendrimers result in high immune responses, whereas the immune response is low without KLH. This indicates that the peptide dendrimer as such is biologically active, but the dendrimer is not a suitable replacement for KLH. Preliminary in vitro investigation on the multivalent binding of a dendrimer with cyclic arginine-glycine-aspartic acid (RGD) residues at the periphery is studied by means of enzyme-linked immunosorbent assay (ELISA). The results indicate an additive effect of the dendrimer. The most promising results are obtained with peptide dendrimers having parts of the β-amyloid peptide at the periphery. The β-amyloid peptide plays an important role in Alzheimer's disease. The peptide dendrimer shows an additive effect on Aβ aggregation and cellular toxicity compared to its monovalent counterpart. Although the multivalent effect is not found in* in-vivo *experiments with CS- and GnRH-conjugated dendrimers, an additive effect of the dendrimer is observed in* in-vitro *investigations using RGD-containing dendrimers and Aβ-conjugated dendrimers. Therefore, the studies presented here give results that stimulate more detailed studies concerning this subject.*

5.1. MULTIVALENCY IN BIOLOGICAL SYSTEMS

Nature uses multivalency to achieve tight binding between proteins and ligands, for example in cell–cell recognition events, where multiple arrays of a ligand on one cell can be recognized by receptors on the opposing cell.[1] Such multivalent arrays of ligands can be accessed by chemical synthesis allowing for the systematic variation of the structure, identity, number and spacing of the ligands.[1-4] Furthermore, chemical synthesis can assist in understanding the mechanism how the multivalent ligands show increased affinity for a receptor as compared to their monovalent counterparts (Figure 5.1).[1,5,6]

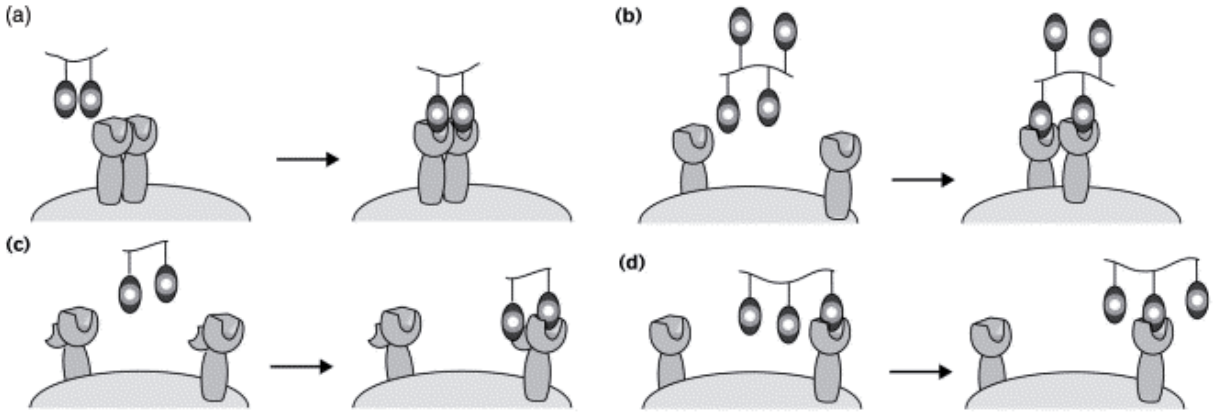


Figure 5.1 *Mechanisms described by Kiessling* et al.[7] *by which multivalent ligands can interact with cell-surface interactions. (a) chelate effect; (b) cluster effect; (c) effect of secondary binding sites; (d) statistical effect.*

One of the mechanisms is described as the chelate effect that uses the binding of the multivalent ligand to oligomeric receptor binding sites (a). Another mechanism is defined as the cluster effect in which receptor sites diffuse towards each other for binding to multiple ligands (b). Binding of ligands to secondary sites next to the primary binding site also results in multivalent binding (subsite effect) (c). Finally, rebinding of the multivalent ligands is favored by the high local concentration of binding elements (statistical effect) (d).[7]

Dendrimers are regarded as ideal candidates for use in biomedical applications, because they are monodisperse, well-defined macromolecules with a globular shape (Chapter 1). For the application of peptide dendrimers in biomedical systems targeting could be enhanced by the multivalent behavior of the specific binding sequences. Therefore, this chapter describes further characterization of the multivalent behavior of the developed peptide dendrimers (Chapter 3) and evaluates their potential in biological applications under physiological circumstances in an initial and preliminary study. For this purpose, research focused on the synthesis and application of four different types of peptide dendrimers. Two *in vivo* and two *in vitro* studies were performed. Both *in vivo* studies were aimed at the development of peptide dendrimers as synthetic vaccines. For a synthetic vaccine against prostate cancer, dendrimers were designed with gonadotropin releasing hormone (GnRH) and tested for

their immune response in rats (section 5.2). Also, dendrimers were fully equipped with asparagine-proline-asparagine-alanine (NPNA) motifs against malaria and were evaluated in rabbits for their immune response (section 5.3). By means of *in vitro* experiments two other applications of peptide dendrimers were investigated. The study of the multivalent nature of *cyclic(*RGDfC)-dendrimer on the binding of integrins was measured using the enzyme-linked immunosorbent assay (ELISA, section 5.4). Finally, dendrimers were modified with four lysine-leucine-valine-phenylalanine-phenylalanine (KLVFF) motifs, which is part of the β-amyloid peptide (Aβ) that plays an important role in Alzheimer's disease. The resulting peptide dendrimer was investigated for its influence in binding to Aβ aggregates (section 5.5).

5.2. PEPTIDE DENDRIMERS AS POSSIBLE SYNTHETIC VACCINES AGAINST PROSTATE CANCER

Peptide dendrimers possessing GnRH derivatives at their periphery are synthesized and tested for their immune response *in vivo* in rats. It is investigated whether peptide dendrimers, being small and well-defined, could be a suitable replacement for the keyhole limpet hemocyanin (KLH) carrier protein. This work is done in close collaboration with dr. P. Timmerman and dr. J.A. Turkstra (Pepscan Systems BV, Lelystad).

5.2.1 INTRODUCTION

Prostate cancer is a significant problem affecting more than ten percent of the male population. Once cancer of the prostate gland occurs, the cancerous cells are usually stimulated to more rapid growth by testosterone. Inhibition of this rapid tumor cell growth can be achieved by removal of both testes so that testosterone cannot be formed. The gonodotropin-releasing hormone (GnRH) is secreted by the hypothalamus and stimulates the anterior pituitary gland to secrete two gonadotropic hormones: (1) luteinizing hormone (LH) and (2) follicle-stimulating hormone (FSH) that in turn stimulates the secretion of testosterone and sperm, respectively. In the absence of GnRH secretion from the hypothalamus, the gonadotropes in the pituitary gland do not secrete either LH or FSH. As a result, no testosterone will be secreted by the testes. Hence, the development of therapeutic vaccines against prostate cancer is based on the inhibition of testosterone secretion. Previously, a multivalency approach was developed by slightly altering and repeating the antigenic peptide motif and a stronger immune response in male pigs (boars) was obtained.[8-10] GnRH has been purified and found to be a decapeptide with peptide sequence: Pyr-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (pEHWSYGLRPG-NH_2).[11]

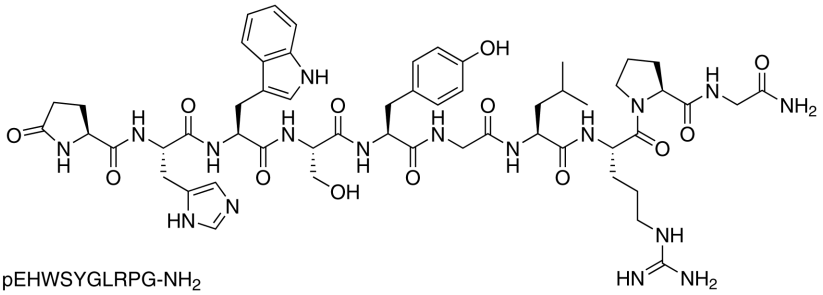


Figure 5.2 *Structural formula of gonodotropin-releasing hormone (GnRH, pEHWSYGLRPG-NH₂).*

Research has been done on the development of synthetic vaccines based on GnRH, in which KLH is frequently used as a carrier protein. A carrier protein is a large molecule capable of stimulating the immune response to the bound ligand. The carrier protein used in this research is KLH. KLH is isolated from the hemolyph of the sea mollusc *Megathura crenulata*[12] and is a high molecular weight copper-containing protein[13] with numerous primary amines available for coupling of haptens.[14]

Where the target hormone GnRH (pEHWSYGLRPG-NH_2) did not give an immune response, the target hormone attached to the carrier protein KLH via the thiol of an extra C-terminal cysteine residue (pEHWSYGLRPGC-KLH) gave an immune response of 40% (40% of the pigs showed an immune response). A "GnRH tandem" peptide sequence (pEHWSYkLRPGQHWSYkLRPGC), in which two glycine residues were replaced with D-lysine residues for attachment to KLH, resulted in an immune response of 80%.[10] In addition, by generating the dimer of this construct via a disulfide bridge of the cysteine residues ("GnRH tandem dimer": $[pEHWSYkLRPGQHWSYkLRPGC]_2$), an immune response of >98% was found. Using this latter sequence, serum testosterone levels were effectively reduced to zero and had a profound effect on testosterone-driven gonodal development.[10]

In this section, dendrimers were used as carriers for the antigenic peptides instead of the KLH-protein. KLH has a high molecular weight, is polydisperse and not well-defined. In contrast, dendrimers are well-defined, monodisperse structures. Therefore, it was hypothesized that they could be a good synthetic alternative for KLH. Peptide dendrimers with the GnRH motif were synthesized using native chemical ligation (Chapter 3) and tested for their immune response in rats.

5.2.2 SYNTHESIS

The thioester peptides were synthesized using *t*Boc-mediated solid phase peptide synthesis SPPS (Chapter 3). The peptide sequence corresponding to GnRH was modified with a *C*-terminal thioester (pEHWSYGLRPG-MPAL **P11**) using the procedure decribed in Chapter 3. Also GnRHtandem was modified with a thioester (pEHWSYGLRPGQHWSYGLRPG-MPAL **P12**) as well as the corresponding peptide sequence with D-lysine residues (pEHWSYkLRPGQHESYkLRPG-MPAL **P13**). These peptide sequences were used to allow the comparison of the results with the data reported in literature.[11]

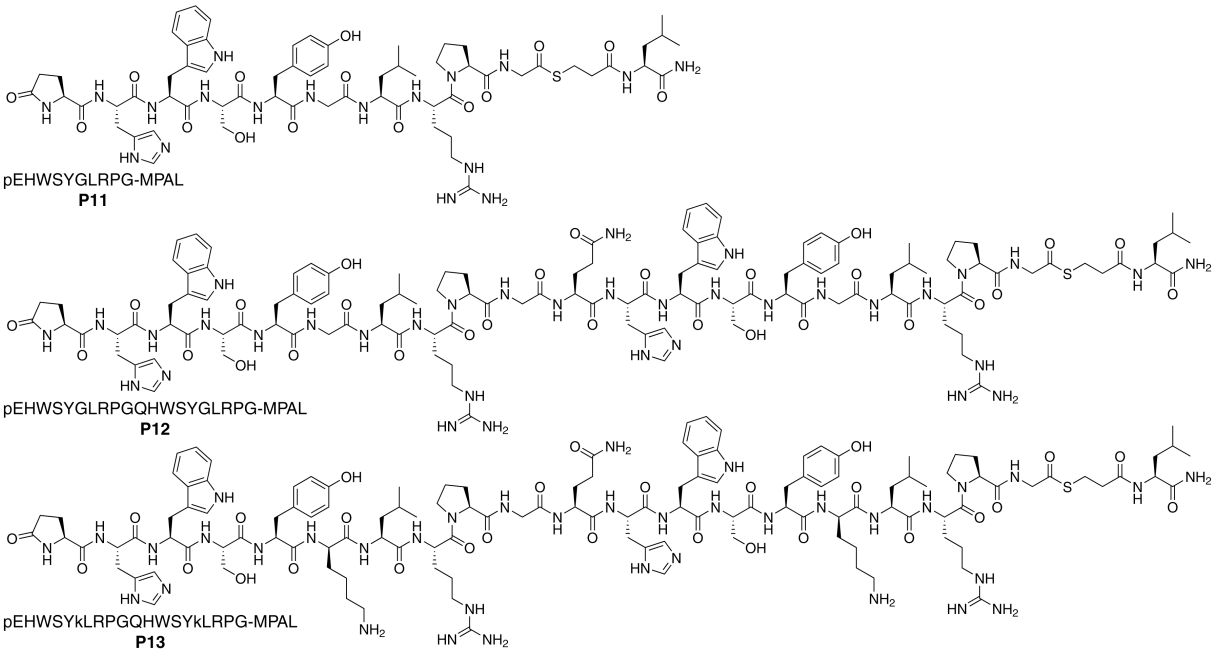


Figure 5.3 *Structural formula of GnRH thioester-peptides **P11**-**P13**.*

The histidine residues in these peptides were protected at the side chain with a 3,5-dinitrophenyl (DNP) group. The DNP group was stable during HF cleavage, but the DNP group was labile under ligation conditions due to the reducing environment. The thioester-peptides **P11-P13** were purified using reversed phase high performance liquid chromatography (RP-HPLC) and analyzed using electrospray ionization mass spectrometry (ESI-MS). The masses found for these peptides corresponded well to the calculated masses (Table 5.1), indicating the desired products were obtained.

Table 5.1 *Observed and calculated masses of thioester-peptides **P11**-**P13**.*

Thioester-peptide		Obs. mass (Da)	Calc. mass (Da)	Yield (%)
pEHWSYGLRPG-MPAL	**P11**	1549.5	1549.6	54% (95 mg)
pEHWSYGLRPGQHWSYGLRPG-MPAL	**P12**	2897.4	2898.0	36% (41 mg)
pEHWSYkLRPGQHWSYkLRPG-MPAL	**P13**	3040.0	3040.3	30-46% (27-49 mg)

GnRH dendrimers were synthesized using standard ligation conditions (Chapter 3). The first generation cysteine dendrimer **D1** was dissolved in a tris(hydroxymethyl)aminomethane (Tris) buffer containing 6 M guanidine and this solution was added to the thioester-peptide. Thiophenol and benzylmercaptan were added (2 v%), the pH was checked to be 7-8 and the reaction was stirred at 37 °C for 1–2 hours. The peptide dendrimer was purified using RP-HPLC and the pure products were obtained in 21–44% yield. The found and calculated masses of the peptide dendrimer products **D1P11**, **D1P12** and **D1P13** corresponded well

(Table 5.2), indicating the desired products were obtained. The ligation product of **P13** with the first generation showed a side product of 2508 Da which could not be identified.
Ligation of thioester-peptide **P13** to second generation cysteine dendrimer **D2** was performed in a similar manner to the ligation of **D1**. The analytical RP-HPLC chromatogram showed complete conversion of the starting material dendrimer **D2**, but the single product peak could not be analyzed on ESI-MS. Therefore, the reaction mixture was washed with diethyl ether to remove excess thiols and the water layer was dialyzed and lyophilized. Again, the single product peak observed in the RP-HPLC chromatogram could not be analyzed on ESI-MS or using MALDI-TOF mass spectrometry, however, the product was subsequently used in the vaccination experiments.

Table 5.2 *Observed and calculated mass of peptide dendrimers.*

Peptide dendrimer		Obs. mass (Da)	Calc. mass (Da)	Yield (%)
$[pEHWSYGLRPGC]_4$-dendrimer	**D1P11**	5390.3	5390.2	21% (7 mg)
$[pEHWSYGLRPGQHWSYGLRPGC]_4$-dendrimer	**D1P12**	10116.9	10119.4	44% (11 mg)
$[pEHWSYkLRPGQHWSYkLRPGC]_4$-dendrimer	**D1P13**	10689.0	10688.4	38% (9 mg)

5.2.3 VACCINATION OF RATS

Male Whistar rats were vaccinated with peptide dendrimers intramuscularly in the left and right hind leg and subcutaneously in the left and right flank. For every dendrimer two rats were sacrificed. The anti-sera (4, 6 and 9 weeks) were both analyzed for the presence of anti-GnRH-dendrimer antibodies as well as for the presence of anti-GnRH antibodies.

5.2.3.1 ELISA AGAINST GNRH-DENDRIMERS

The results of the ELISA against GnRH dendrimers are shown in Table 5.3. Four peptide dendrimers (**D1P11**, **D1P12**, **D1P13**, and **D2P13**) were tested as well as two controls, all without the carrier protein KLH. Immune responses were measured 12 weeks post vaccination. Two controls were measured: GnRHtandemdimer(k) and GnRHtandemdimer. GnRHtandemdimer(k) was known to show a high immune response (section 5.2.1), but only when attached to KLH. When not coupled to KLH, it showed a low response (Table 5.3). Therefore, GnRHtandemdimer was also measured as a control; it showed high response. The immune response of the peptide dendrimers must be similar to this value in order for it to be suitable as a synthetic vaccine against prostate cancer. However, the peptide dendrimers **D1P11**, **D1P12**, **D1P13** and **D2P13** showed a low immune response compared to the control GnRHtandemdimer.

Table 5.3 *Immune responses measured against GnRH dendrimers.*

Peptide dendrimer	Immune response rat 1	Immune response rat 2
D1P11	--	--
D1P12	+	-
D1P13	--	--
D2P13	-	--
GnRHtandem(k)	--	--
GnRHtandem	++	++

5.2.3.2 ELISA AGAINST GNRH

The sera were also tested against GnRH peptides corresponding to the GnRH-dendrimers with which the rats were vaccinated (data not shown). Immune responses were measured 10 weeks post vaccination. The control GnRHtandemdimer(k) showed a high response, and GnRH tandem dimer showed a mediate response. The vaccination of peptide dendrimers resulted in all cases in a low immune response compared to the control GnRHtandemdimer.

Table 5.4 *Immune responses measured against GnRH.*

Peptide dendrimer	Immune response rat 1	Immune response rat 2
D1P11	-	-
D1P12	+	-
D1P13	-	-
D2P13	-	-
GnRHtandem(k)	++	++
GnRHtandem	+	+

According to literature, hydrophobicity[15] and preorganization[16] play an important role in the induction of an immune response. Compared to KLH, the dendrimers is smaller, more flexible and less hydrophobic. These features could contribute to the low immune responses observed. In future, conformational studies should be performed to give evidence about the actual preorganization of GnRH at the dendrimer periphery.

In summary, the data showed that little or no immune responses were found in rats vaccinated with peptide dendrimers. Since the same peptides attached to KLH were active components to obtain an immune response, it was hypothesized that the sizes of the dendrimers were too small compared to KLH or the conformation of the peptides at the periphery of the dendrimer was unfavorable for inducing an immune response. In order to test the generality of this phenomenon, peptide dendrimers were tested as synthetic vaccines against malaria (section 5.3). In this case, the immune responses of peptide dendrimers were compared to the results of the peptide dendrimer-KLH constructs.

5.3. PEPTIDE DENDRIMERS AS POSSIBLE SYNTHETIC VACCINES AGAINST MALARIA

First and second generation peptide dendrimers with a asparagine-proline-asparagine-alanine (NPNA) motif at their periphery were synthesized and tested for their immune response in rabbits. In the previous section it seemed that dendrimers did not induce an immune response. Here, it was evaluated whether the peptide dendrimer as such was biologically active by comparison of the immunization of peptide dendrimer with the immunization of the corresponding peptide dendrimer-KLH construct. For this purpose, peptide dendrimers were tested as synthetic vaccines against malaria. A first impression of the conformation of the peptides was obtained by performing CD spectroscopy. This work was done in close collaboration with dr. D.W.P.M Löwik, J.T. Meijer and A.M. Kaan (Radboud University Nijmegen).

5.3.1 INTRODUCTION

Malaria represents one of the world's most serious health problems, with hundreds of millions of people affected worldwide.[17] *Plasmodium falciparum* is the most virulent of the plasmodial parasites that cause malaria. Infection of human hosts is initiated by the sporozoite stage, which is injected into the circulation following attack by infested mosquitoes. The sporozoites migrate to the liver where a specific ligand–receptor interaction, mediated by one of the major surface membrane proteins on the parasite, the circumsporozoite (CS) protein, may lead to invasion of liver cells.[18]

The development of a malaria vaccine is based on the observation that antibodies against the CS protein were able to block infection by sporozoites.[19,20] This immunoresponse is directed against an NPNA motif which is repeated almost 40 times in the central region of the CS protein.[20-23] The conformation of the NPNA repeat region in the intact CS protein appeared to be of importance. Based on sequence preferences of β-turns in protein crystal structures,[24] a type–I β-turn seems a likely conformation for the NPNA motif in an aqueous solution.[25] Research has been done on the design of a synthetic vaccine based on the NPNA motif that stabilizes the β-turn, *e.g.* by attaching the peptide sequence to a template,[26-28] or by modifying it with apolar tails for stabilization in liposomes.[29,30]

In the current investigation, dendrimers were synthesized with the NPNA motif at their periphery. In the previous section the immune response found for peptide dendrimers was low. Here, it was investigated whether the peptide dendrimer as such is biologically active by comparison of the result of the peptide dendrimer with the results of the corresponding peptide dendrimer-KLH construct. Rabbits were vaccinated with these conjugates and the amount of antibody present in the sera of the rabbits was measured using a spot-test (see experimental section).[31,32]

5.3.2 SYNTHESIS

Four thioester-peptides comprising the NPNA sequence were synthesized using *t*Boc-mediated SPPS (Chapter 3). Four different peptide sequences **P14**, **P15**, **P16** and **P6** were synthesized that have different properties regarding the maintenance of the β-hairpin structure. The design was such that this stabilization might be achieved by either by the formation of disulfide bridges (**P15**), additional interactions for back folding of the *N*-terminus to the interior of the dendrimer (**P6**) or forced loop structures (**P17**). Using *t*Boc-mediated SPPS, the peptides **P14-P16** and **P6** were all obtained in 36-76% yield (30-56 mg, Table 5.5).

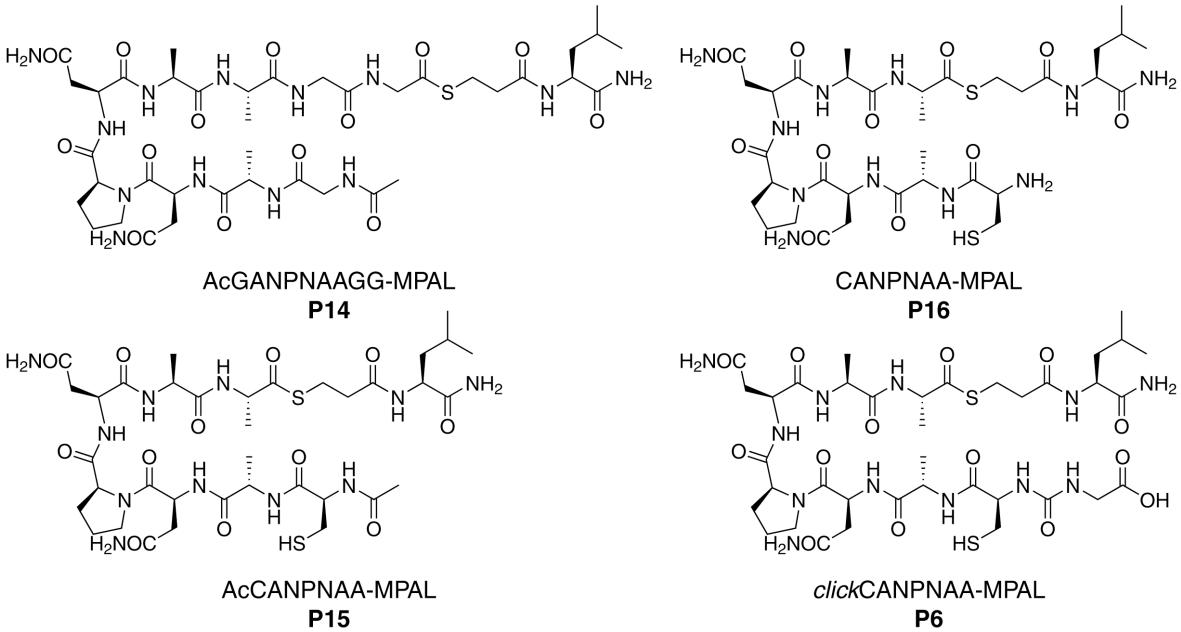
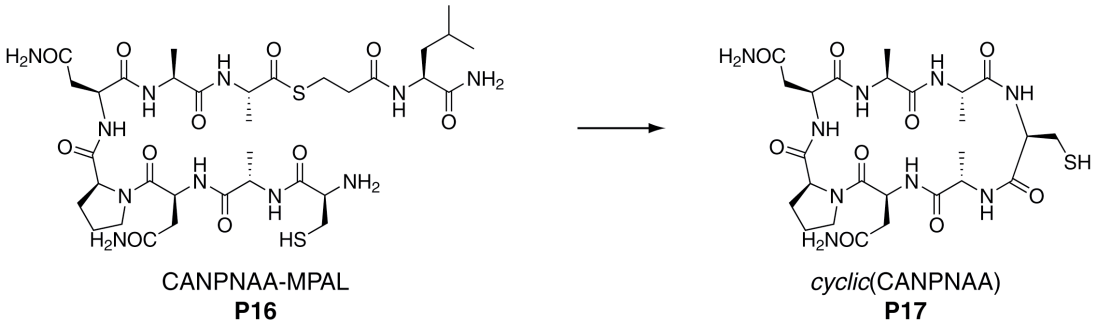


Figure 5.4 *Thioester-peptides **P14-P16** and **P6** synthesized via* t*Boc-mediated solid phase peptide synthesis.*

The thioester peptide CANPNAA-MPAL **P16** was cyclized to **P17** by performing an intramolecular ligation of the *N*-terminal cysteine with the *C*-terminal thioester of the peptide (Scheme 5.1). This resulted in the *cyclic*(CANPNAA) peptide **P17**. The peptide **P16** was dissolved in a Tris buffer at pH 8.0 containing 6 M guanidine. Thiols were added for thioester exchange and after 1 hour of reaction time, the peptide **P17** was purified using RP-HPLC and obtained in 76% yield (Table 5.5).



Scheme 5.1 *Synthesis of cyclic(CANPNAA) **P17** from CANPNAA-MPAL **P16**.*

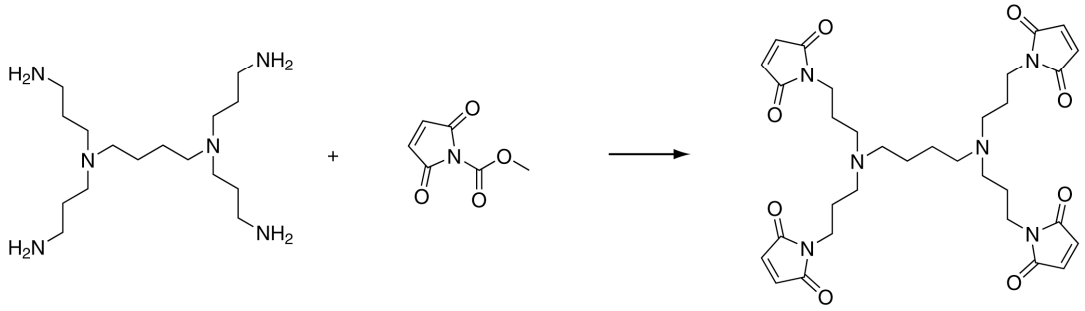
Table 5.5 *Found and calculated masses of (thioester-)peptides synthesized.*

Thioester-peptide		Obs. mass (Da)[a]	Calc. mass (Da)	Yield (%)
AcGANPNAAGG-MPAL	**P14**	970.3	970.0	43% (50 mg)
AcCANPNAA-MPAL	**P15**	902.4	902.0	75% (30 mg)
CANPNAA-MPAL	**P16**	860.2	860.0	47% (56 mg)
*click*CANPNAA-MPAL	**P6**	961.3	961.0	36% (48 mg)
cyclic(CANPNAA)	**P17**	642.4	641.7	76% (26 mg)

[a] $[M+H]^+$ masses are shown.

The corresponding peptide dendrimers were synthesized using native chemical ligation conditions (Chapter 3). For that purpose, the first generation dendrimer **D1** was dissolved in Tris buffer at pH = 8.0 containing 6 M guanidine and added to four equivalents of the peptide (**P14**, **P15** or **P6**). Thiophenol and benzylmercaptan were added for thioester exchange and after 1 hour of reaction time, the peptide dendrimer was purified using RP-HPLC and the pure peptide dendrimers **D1P14**, **D1P15** and **D1P6** were obtained in 29%, 43% and 20% yield, respectively. As discussed in Chapter 3, the differences in observed and calculated mass for **D1P6** is likely to originate form disulfide bonds in the product (Table 5.6). Similar conditions were used for the synthesis of **D2P14**. To the second generation cysteine-dendrimer **D2** eight equivalents of **P14** was added and the pure peptide dendrimer was obtained in 32% yield (Table 5.6).

The free thiol of the cyclic peptide **P17** is able to react with a maleimide (Chapter 4). For that reason, a first generation dendrimer with maleimide groups at the periphery was synthesized by reaction of a first generation poly(propylene imine) dendrimer with methoxycarbonylmaleimide (Scheme 5.2). The product **DM1** was obtained as a yellow powder in 74% yield and analyzed using ^{1}H NMR spectroscopy and ESI-MS.



Scheme 5.2 *Synthesis of a first generation of [maleimide]$_4$-dendrimer **DM1**. Reaction conditions: THF, $NaHCO_3$ (aq, sat).*

Table 5.6 *Observed and calculated masses of peptide dendrimers synthesized.*

Peptide dendrimer		Obs. mass (Da)	Calc. mass (Da)*	Yield (%)
[AcGANPNAAGGC]$_4$-dendrimer	**D1P14**	3735.2	3736.1	29% (6 mg)
[AcGANPNAAGGC]$_8$-dendrimer	**D2P14**	7611.3	7612.4	32% (6 mg)
[AcCANPNAAC]$_4$-dendrimer	**D1P15**	3464.1	3464.0	43% (12 mg)
[*click*CANPNAAC]$_4$-dendrimer	**D1P6**	3694.8	3700.1	20% (9 mg)
[maleimide]$_4$-dendrimer	**DM1**	637.3	636.7	74 % (188 mg)
[*cyclic*(CANPNAA)]$_4$-dendrimer	**DM1P17**	3203.0	3203.5	42% (12 mg)

*reduced mass

The cyclic peptide **P17** was coupled to the resulting first generation maleimide dendrimer **DM1** by reaction in Tris buffer of pH 7. The peptide dendrimer **DM1P17** was purified using RP-HPLC and obtained in 42% yield (Table 5.6).

5.3.3 DETERMINATION OF SECONDARY STRUCTURE

In order to study the secondary structure of the synthesized peptide dendrimers, CD spectra are recorded. For proteins, a change in the CD spectrum can directly be related to a change in conformation and different CD effects are observed for a random coil, α-helix or a β-sheet conformation.[30,33-35] However, for peptides this is not necessarily the case, as peptides are small molecules, single amino acids can have influence on the CD effect of the compound. In addition, peptides that are in a β-turn (as expected for the NPNA motif) do not always show a β-sheet content in the CD spectrum. Nevertheless, to get a first impression about the conformation of the NPNA motif in the peptide dendrimers, CD spectra are recorded of the dendrimers in aqueous solutions.

The obtained spectra are compared to a polylysine model. Polylysine in a random coil conformation shows an absorption minimum at 197 nm. The α-helix conformation shows a maximum at 191 nm and two minima at 209 nm and 222 nm. Polylysine in a β-sheet conformation shows a maximum at 195 nm and a minimum at 216 nm. The data obtained for the peptide dendrimers investigated are compared to this model system.

In the CD spectra of peptide dendrimers **D1P14**, **D2P14**, **D1P15** and **D1P6** an absorption minimum was observed at 200 nm, indicating random coil conformation of the peptides. For peptide dendrimer **DM1P17** an absorption maximum was observed at 190 nm as well as a minimum at 205 nm, indicating β-sheet conformation of the peptides. This difference in conformation can be explained by the fact that the peptide in **DM1P17** was cyclic and as a result the peptides were forced to be in a loop structure.

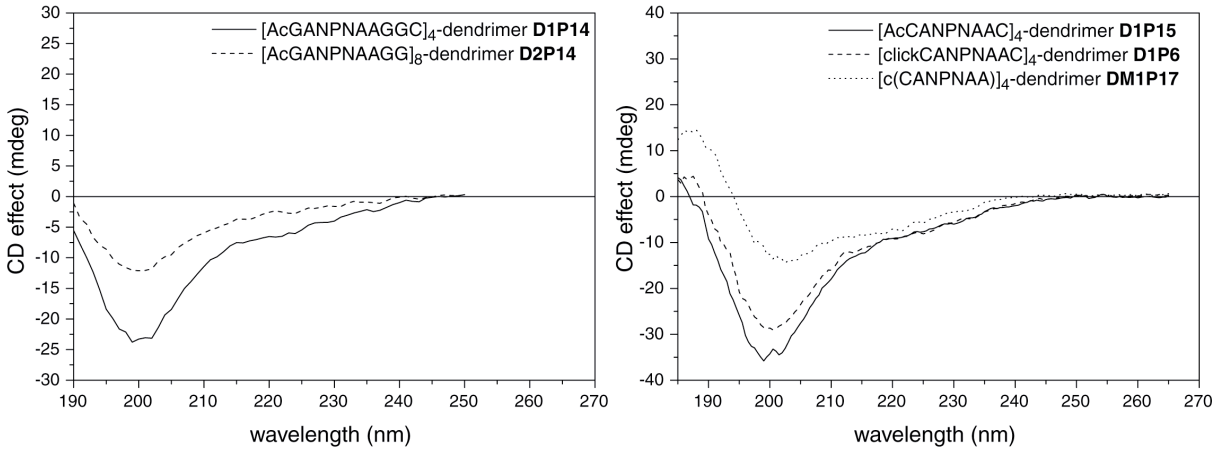


Figure 5.5 *CD spectra of* ***D1P14*** *and* ***D2P14*** *(left),* ***D1P15****,* ***D1P6*** *and* ***DM1P17*** *(right) at a concentration of 0.2 mg/mL.*

Assuming that in peptides β-sheet content, as determined by the CD effect, was related to β-turn content, the results indicated that the peptides in **DM1P17** were preferably in a β-turn, whereas the peptides in the peptide dendrimers **D1P14**, **D2P14**, **D1P15**, and **D1P6** were in a random coil conformation.

5.3.4 VACCINATION OF RABBITS

The peptide dendrimers were tested for their immune response *in vivo* by vaccination in rabbits. For that purpose, peptide dendrimer conjugates with a carrier protein were also synthesized to test the effect of the dendrimer. For coupling of peptide dendrimers to the carrier protein KLH, the coupling agent *N*-succinimidyl-6-maleimidocaproate (MHS) was used. The activated ester of MHS was reacted with the amines of the lysine residues in KLH, resulting in a maleimide-functionalized KLH construct: KLH*. Subsequently, the thiols of the cysteine residues in peptide dendrimers were reacted with the maleimides of KLH*, resulting in peptide dendrimer-KLH constructs. Solutions of these constructs were used for vaccination in rabbits (experimental section). Rabbits were vaccinated in the lymph node and boost injections were done subcutaneously. Two rabbits were sacrificed per peptide dendrimer construct. Blood samples were taken regularly and with the serum of these blood samples, a spot-test was performed (experimental section).

Clear immune responses in both rabbits were found for **D1P14-KLH**, **D1P15-KLH**, and **D1P6-KLH**. The vaccination of rabbits with **D2P14-KLH** resulted in an immune response in only one rabbit. This is unexpected as the second generation dendrimer (**D2P14-KLH**) is not expected to have lower immune responses than the first generation (**D1P14-KLH**). For **D1P15** and **DM1P17** low immune responses were found.

Table 5.7 *Results of the spot-test of the vaccination of peptide dendrimers.*

Peptide dendrimer construct	Immune response rabbit 1	Immune response rabbit 2
D1P14-KLH	++	++
D2P14-KLH	+	--
D1P15	-	--
D1P15-KLH	++	++
D1P6-KLH	++	++
DM1P17	--	--

Coupled to KLH, all peptide dendrimers showed an immune response after vaccination in rabbits. However, without KLH as a carrier protein, no immune response was found. These results were in agreement with the results obtained for the vaccination of rats with GnRH-conjugated dendrimers (section 5.2). The observation that the peptide dendrimer-KLH conjugate showed an immune response indicated that the peptide dendrimer as such was biologically active, but that the dendrimer was not a suitable replacement for KLH.

5.4. PEPTIDE DENDRIMERS IN MULTIVALENT BINDING TO INTEGRINS

As a model system for multivalent binding of dendrimers, the binding of a RGD-containing dendrimer to integrin $\alpha_V\beta_3$ is investigated *in vitro* using enzyme-linked immunosorbent assay (ELISA) and the results are compared to its monovalent counterpart. This work is done in close collaboration with E.H.M. Lempens (Eindhoven, University of Technology).

5.4.1 INTRODUCTION

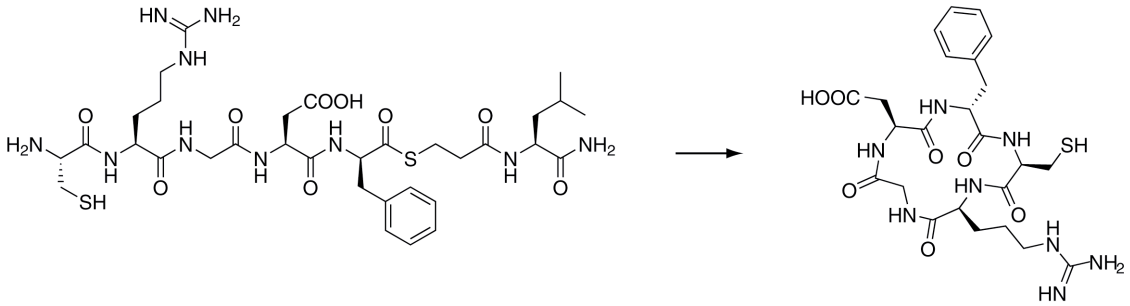
The arginine-glycine-aspartic acid (RGD) peptide motif present in fibronectins and vitronectins has been identified as a universal integrin recognition element. It is reported in the literature that *cyclic*(RGDfX) peptides have a lower dissociation constant (K_d ~ 1-10 nM) in binding to integrin $\alpha_V\beta_3$ than their linear counterparts (K_d ~ 1 μM).[36] Multivalent RGD constructs have been synthesized aiming for inhibition of $\alpha_V\beta_3$ integrin-binding to their natural ligands, serving as anti-angiogenic drugs.[37-41] Kessler *et al.* have stressed the importance of the spacer length between the RGD peptide sequence and the carrier. *In vitro* investigations revealed that osteoblasts do not attach to surfaces containing *cyclic*(RGDfK) coupled via a 8-atom spacer, while a 15-atom spacer did promote attachment. Hence, in the current investigation, the multivalent binding of a *cyclic*(RGDfC)-containing dendrimer with an 15-atom spacer to an integrin-coated surface was investigated.

5.4.2 SYNTHESIS

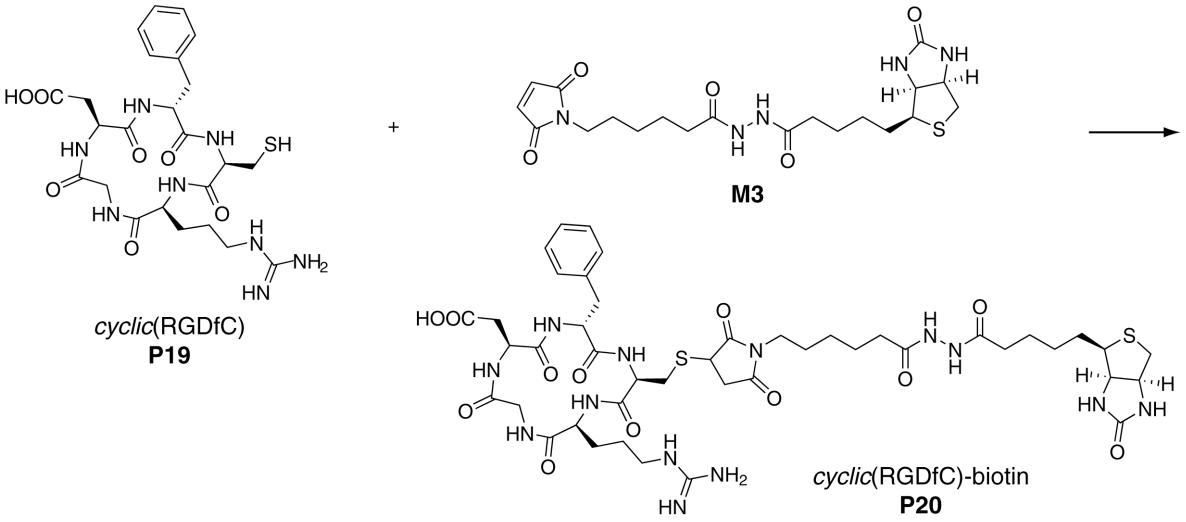
For the synthesis of *cyclic*RGD peptide, the corresponding linear peptide sequence with a *C*-terminal thioester was synthesized using *t*Boc-mediated SPPS, as was described in Chapter 3.

After purification using RP-HPLC, the pure peptide **P18** was obtained in 39-54% yield. To obtain the *cyclic*RGD peptide **P19**, **P18** was cyclized *via* intramolecular native chemical ligation (Scheme 5.3). For that purpose, the peptide **P18** was dissolved in guanidine buffer at pH = 8.0. Thiophenol and benzylmercaptan were added for thioester exchange and the reaction was monitored using RP-HPLC. Next to the cyclic peptide **P19**, also small amounts of the cyclic dimer and trimer of the peptide were observed. When the reaction was performed at lower concentrations of peptide, no change in the ratio of monomer, dimer and trimer was observed. The monomeric cyclic peptide **P19** was purified using RP-HPLC and isolated in 27-39% yield.

The peptide was biotinylated to allow the use of ELISA[42] for evaluating the binding to integrin $\alpha_V\beta_3$. The biotin label was introduced at **P19** through a maleimide reaction of biotin (**M3**) with the thiol of the cysteine residue that became available after the native chemical ligation reaction (Scheme 5.4, for an extensive investigation of the applicability of this reaction for peptide dendrimers, the reader is referred to Chapter 4). The peptide **P19** was dissolved in Tris buffer of pH = 7.0 and maleimide-biotin was added. After 1 hour, the peptide **P20** was purified using RP-HPLC and obtained in 30-46% yield.



Scheme 5.3 *Synthesis of cyclic(RGDfC)* ***P19*** *by intramolecular native chemical ligation. Reaction conditions: 2 v% thiophenol, 2 v% benzylmercaptan, Tris buffer containing 6 M guanidine.*



Scheme 5.4 *Synthesis of cyclic(RGDfC)-biotin* ***P20****. Reaction is performed in a Tris buffer at pH 7.*

For the synthesis of a peptide dendrimer with a cyclic RGD peptide sequence, native chemical ligation cannot be performed, as the peptide lacks an *N*-terminal cysteine or a *C*-terminal thioester. Hence, a maleimide-functionalized thioester was synthesized using *t*Boc-mediated SPPS (Figure 5.6). In addition, three glycine units were incorporated resulting in a short spacer between the RGD binding unit and the dendrimer scaffold. The thioester was coupled to the resin (Chapter 3) and three glycine residues were introduced. Subsequent addition of maleimide-β-alanine and cleavage of the resin using HF, resulted in maleimide-GGG-MPAL **M8**. The product was purified using RP-HPLC and analysis using ^{1}H NMR, ^{13}C NMR and ESI-MS revealed that the pure product was obtained.

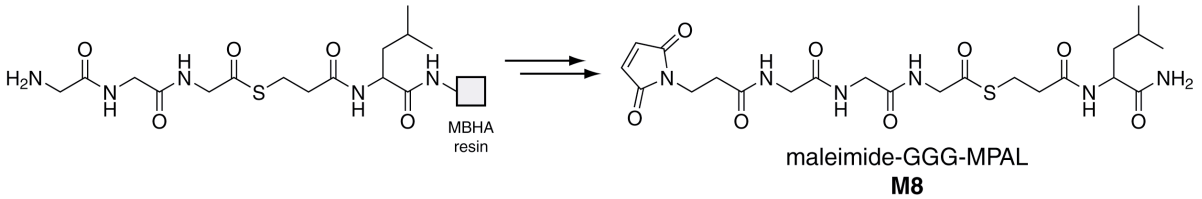
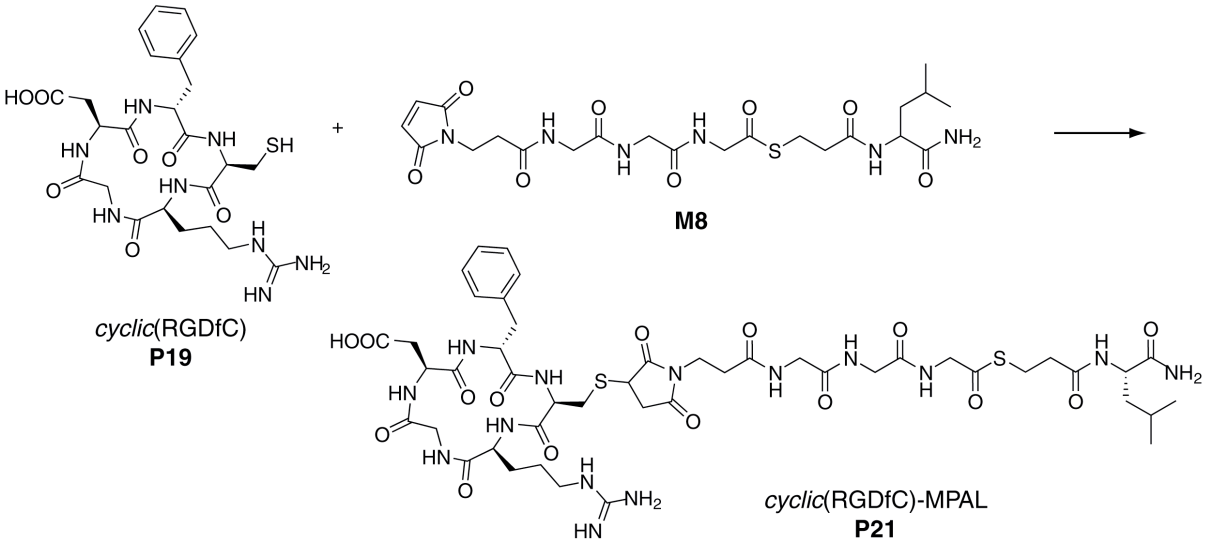


Figure 5.6 *Synthesis of maleimide-GGG-MPAL **M8** using* t*Boc-mediated solid phase peptide synthesis as described in Chapter 3 of this thesis.*

Maleimide-GGG-MPAL **M8** was introduced to **P19** via maleimide-thiol reaction (Scheme 5.5, Chapter 4). The peptide **P19** was dissolved in 0.1 M Tris buffer of pH = 7.0, **M8** was added and after 2 hours the peptide **P21** was purified using RP-HPLC and obtained in 77% yield.



Scheme 5.5 *Synthesis of cyclic(RGDfC)GGG-MPAL. Reaction was performed in Tris buffer at pH = 7.0 for 2 hours.*

The peptide **P21** was subsequently introduced to the dendrimer scaffold via native chemical ligation. **P21** was dissolved in Tris buffer at pH = 8.0 containing 6 M guanidine and **D1** was added. Thiophenol and benzylmercaptan were added for thioester exchange and after 1 hour, the peptide dendrimer **D1P21** was purified using RP-HPLC and isolated in 28% yield.

Table 5.8 *Observed and calculated mass of peptides and peptide dendrimers.*

Peptide(-dendrimer)		Obs. mass (Da)	Calc. mass (Da)	Yield (%)
CRGDf-MPAL	**P18**	797.6	796.9	39-54%
cyclic(RGDfC)	**P19**	579.3	578.6	27-39%
cyclic(RGDfC)-biotin	**P20**	1029.4	1030.1	13%
MaleimideGGG-MPAL	**M8**	541.3	540.5	*c.p.*[a]
cyclic(RGDfC)-MPAL	**P21**	1118.6	1119.2	77%
[*cyclic*(RGDfC)]$_4$-dendrimer	**D1P21**	4332.5	4332.8	28%

a) c.p. = crude product

5.4.3 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The multivalent binding of the peptide dendrimer to the integrin $\alpha_v\beta_3$ was quantified using enzyme-linked immunosorbent assay (ELISA) technique.[42] First, the dissociation constant of *cyclic*(RGDfC)-biotin **P20** was determined. The subsequent steps in the direct ELISA performed here were the coupling of integrin $\alpha_v\beta_3$ to a polystyrene solid support, incubation with *cyclic*(RGDfC)-biotin **P20**, washing and addition of an enzyme-labeled antibody. After that, the substrate 3,3',5,5'-tetramethylbenzidine (TMB) was added that is converted into a colored product by the enzyme. The change in absorption at 450 nm vs. the concentration of **P20** could be fitted with a sigmoidal curve (Figure 5.7). The fit through the average value of the two measurements gave a dissociation constant K_d of 20 ± 4 nM. This value is in agreement with literature values.[38]

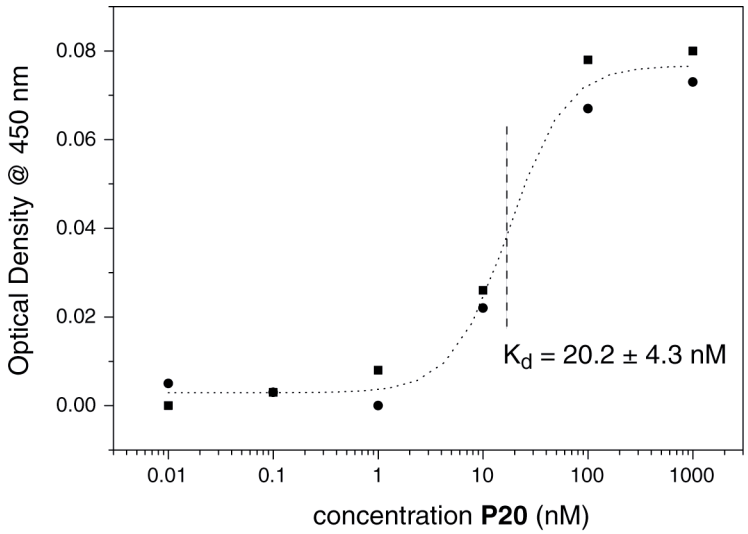


Figure 5.7 *Optical density at 450 nm plotted against the logarithmic concentration of **P20**. The experiment was performed in duplicate (shown in the graph with solid circles • and squares ■), the dotted line represents the fit through the average values of the two experiments using Equation 4 (experimental section), from which the dissociation constant K_d is determined.*

5.4.4 COMPETITIVE ELISA

The competitive ELISA differs from the direct ELISA described above. In the incubation step of integrin $\alpha_V\beta_3$ with the peptide *cyclic*(RGDfC)-biotin **P20**, an additional peptide is added, resulting in competitive binding of the two RGD-peptides to integrin $\alpha_V\beta_3$. In this way, a competition experiment was performed between the monovalent *cyclic*(RGDfC)-biotin **P20** and the corresponding multivalent [*cyclic*(RGDfC)]$_4$-dendrimer **D1P21**. These results were compared to the results of a competition experiment between *cyclic*(RGDfC)-biotin **P20** and *cyclic*(RGDfC) **P19** (both monovalent). The concentration of **P20** was constant in all measurements and the optical densities obtained were plotted against the concentration of peptide that had performed competitive binding with **P20**.
An ELISA of **P20** in presence of **P19** showed that competition takes place for the available binding sites on integrins. Using **D1P21** as inhibitor, the optical density seemed to drop faster (at lower concentrations) than in case of **P19**. The K_d of **D1P21** might therefore be lower than the K_d of **P19**, indicating a possible multivalent effect. However, no reliable quantitative comparison can be made, because of large errors in the measurements and difficulties in reproducibility.

5.5. PEPTIDE DENDRIMERS TO AFFECT AGGREGATION AND TOXICITY OF β-AMYLOID PEPTIDE IN ALZHEIMER'S DISEASE

The aggregation and toxicity of β-amyloid peptide (Aβ) play an important role in Alzheimer's disease. Part of the Aβ peptide is attached to a first generation dendrimer and a preliminary *in vitro* study is performed to investigate the effect of the peptide dendrimer on Aβ aggregation and toxicity. This work is done in close collaboration with dr. W. Scheper and S.M. Chafekar (Neurogenetics lab, Academic Medical Center, University of Amsterdam).

5.5.1 INTRODUCTION

Alzheimer's disease is characterized by progressive memory loss and other cognitive and behavioral deficits and is the most common cause of dementia.[43] It is a prime example of a protein folding disease.[44] An important event in the pathogenic cascade of Alzheimer's disease is the formation of the highly fibrillogenic β-amyloid peptide (Aβ), by proteolytic cleavage of the amyloid precursor protein (APP).[45] According to the amyloid cascade hypothesis, Aβ is the primary cause of Alzheimer pathogenesis, and an imbalance in the processing of APP is the initial event leading to plaques and tangles.[46-49] Aβ has a high propensity to aggregate and readily forms small oligomeric aggregates that can progress to highly structured aggregates (fibrils) in a dynamic process. Aggregation of Aβ seems a prerequisite for toxicity, as monomers are apparently not toxic.[50] The severity of dementia of the patients suffering from Alzheimer's disease does not correlate with the plaque load in the brain,[51,52] which at first glance seems to contradict the amyloid cascade hypothesis. However,

recent reports suggest that oligomeric aggregates are toxic, rather than the fibrils, which may serve to sequester the toxic protein aggregates.[50,53,54] Prevention of Aβ aggregation, consequently, has emerged as a potential goal in the therapy or prevention of Alzheimer's disease.[55]

Previous studies indicate that Aβ polymerization *in vivo* and *in vitro* is a specific process that involves interactions between binding sequences in the Aβ peptide.[56-58] A rational pharmacological approach for prevention of amyloid formation would therefore be to use drugs that specifically interfere with Aβ–Aβ interaction and polymerization. Several new ligands or ligand classes have been identified which interfere with fibril or plaque formation.

Tjernberg was the first to discover that peptides incorporating a short Aβ fragment (KLVFF; $A\beta^{16-20}$) can bind full-length Aβ and prevent its aggregation into amyloid fibrils.[59] The data suggest that KLVFF serves as a binding sequence during Aβ polymerization and fibril formation. Moreover, the KLVFF peptide may serve as a lead compound for the development of peptide and non-peptide agents aimed at inhibiting Aβ amyloidogenesis *in vivo*. As a result, several other research groups investigated in more detail the influence of KLVFF on Aβ aggregation.[60,61]

Colloidal gold particles have been used previously to serve as a scaffold to couple multiple Aβ molecules.[62] It was investigated whether the resulting molecule could serve as a mimic for Aβ. A disadvantage of this approach is that the size of the particles and the number of peptide attached to it can not be controlled. These problems are not encountered using dendrimers. In this project dendrimers are not used as a mimic of Aβ, but are investigated as possible inhibitors of Aβ aggregation and toxicity. For this purpose, the pentapeptide KLVFF was coupled four times to a first generation poly(propylene imine) dendrimer (**D1**) via native chemical ligation (Chapter 3). The ability of the resulting peptide dendrimer to inhibit β-sheet formation and decrease toxicity of Aβ(1-42) was studied *in vitro*.

5.5.2 SYNTHESIS

In this study, the following peptides were used; AcKLVFF-C6-*click* **P22**, KLVFF-C6-*click* **P22'**, and AcKLVFFGG-MPAL **P5** and KLVFFGG-MPAL **P5'**, (KLVFF corresponds to Aβ(16-20)). The *C*-terminal thioester-peptides **P5** (Chapter 3) and **P5'** were synthesized using *t*Boc-mediated SPPS (Chapter 3). The peptides were purified using RP-HPLC and analysis using ESI-MS revealed that the desired products were obtained.

Native chemical ligation was performed using 1 equivalent of **D1** and 4 equivalents of **P5'**. Thiophenol and benzylmercaptan were added for thioester exchange (pH = 7.5) and the mixture was vigorously stirred. After 1 hour of reaction time, the peptide dendrimer was purified using RP-HPLC. The peptide dendrimer was analyzed using ESI-MS. The observed masses and calculated masses of **P5** (Chapter 3), **P5'** and **D1P5'** corresponded well (Table 5.9), indicating the desired products were obtained.

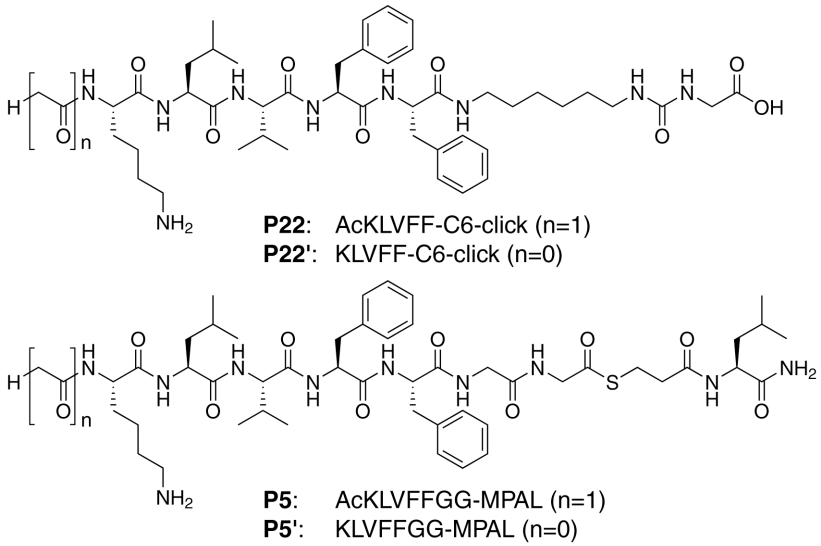


Figure 5.8 *(Thioester-)peptides synthesized using* t*Boc-mediated solid phase peptide synthesis, KLVFF corresponds to Aβ(16-20).*

Table 5.9 *Observed and calculated mass of peptide and corresponding peptide dendrimer.*

Peptide(-dendrimer)		Obs. mass (Da)	Calc. mass (Da)	Yield (%)
KLVFFGG-MPAL	**P5′**	967.5	967.2	58%
$[KLVFFGGC]_4$-dendrimer	**D1P5′**	3725.2	3724.8	25%

5.5.3 EFFECT OF KLVFF-DENDRIMER ON Aβ AGGREGATION AND TOXICITY

The peptide dendrimer and peptides were tested for their ability to interfere with aggregation of Aβ(1-42). To visualize the fibrils and oligomers formed, electron microscopy (EM) was used. To investigate the β-sheet content of the fibrillar structures formed, a thioflavine T fluorometric assay was performed (experimental section)[63] and an MTT test was performed to assess the toxicity of Aβ(1-42) aggregates on differentiated neuroblastoma cells. Aβ(1-42) preparations enriched in fibrillar (Figure 5.9b) or oligomeric (Figure 5.9a) Aβ can be made, depending on the conditions used (experimental section). As expected, oligomeric Aβ is more toxic than fibillar Aβ. The fibrils of Aβ(1-42) show high thioflavine fluorescence emission at 492 nm (typical of amyloids), whereas this fluorescence for the oligomers of Aβ(1-42) is low. The controls **P22′** and **D1P5′** (without Aβ(1-42)) also did not show the typical amyoid thioflavine fluorescence emission.

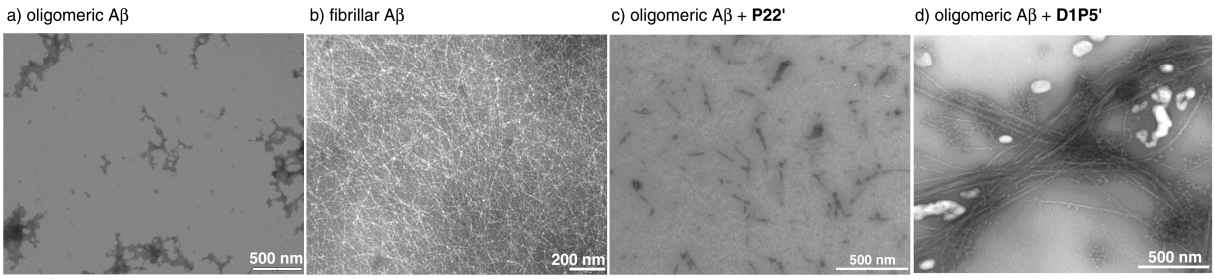


Figure 5.9 *Representative EM pictures of oligomeric and fibrillar Aβ(1-42). In the presence of the KLVFF peptide* ***P22′*** *larger structures are formed, an effect much stronger for the KLVFF dendrimer* ***D1P5′****.*

The acetylated peptides **P22** and **P5** did not affect the aggregation of Aβ(1-42). Therefore the influence of the peptide dendrimer **D1P5** was not further investigated.

The incubation of the peptide **P22′** and peptide dendrimer **D1P5′** (both with a free *N*-terminus) with Aβ under fibril-forming conditions resulted in no changes in the morphologies of the fibrils. However, the fibrillar aggregates obtained showed a high thioflavine fluorescence signal (Figure 5.10) compared to fibrils of Aβ(1-42) alone.

P22′ and **D1P5′** were also incubated with Aβ(1-42) under oligomer-forming conditions. For the incubation of **P22′** (250 μM) with Aβ (100 μM) in a 2.5-fold molar excess, small fibrillar aggregates were obtained (Figure 5.9c). These fibrillar aggregates showed similar β-sheet content in the thioflavine assay as compared to oligomers of Aβ alone, indicating that the fibrils formed have a different morphology than the fibrils obtained from aggregation of Aβ alone.

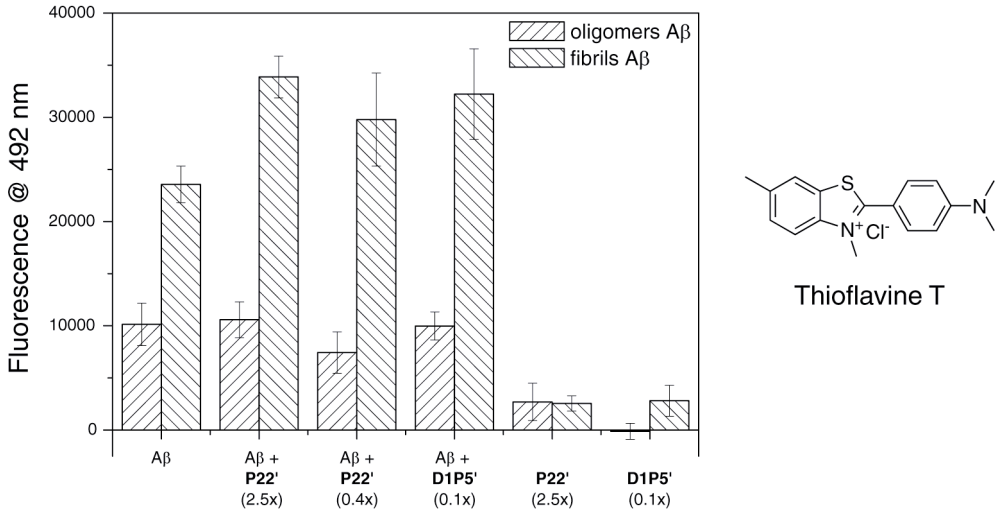


Figure 5.10 *Thioflavin T Fluorescence Assay determined for Aβ(1-42) with different amounts of* ***P22′*** *and* ***D1P5′****.*

The influence of the peptide dendrimer having four KLVFF motifs **D1P5′** on the aggregation behavior of Aβ under oligomer-forming conditions was studied. Large and thick fibrils were obtained when **D1P5′** (62.5 μM) was added to Aβ (100 μM) in a 2.5-fold molar excess of KLVFF (Figure 5.9d). Also in this case, the obtained fibrillar aggregates showed similar β-

sheet content in the thioflavine assay as compared to oligomers of Aβ alone, indicating that the fibrils formed have a different morphology than the fibrils obtained from aggregation of Aβ alone (Figure 5.10).

In contrast to what was expected, the peptide and peptide dendrimer do not inhibit aggregation of Aβ(1-42), but induce aggregation in a fibrillar manner that is different in morphology form the fibrils of Aβ alone. It is now interesting to see what the effect of the peptide and peptide dendrimer is on toxicity of Aβ(1-42). In addition to the electron microscopy analysis and thioflavin T assay, the toxic effect of the samples was tested using differentiated SK-N-SH human neuroblastoma cells. To induce neural differentiation, cells were cultured for 5 days in the presence of all-*trans* retinoic acid. Because under fibrillar conditions, no inhibitory effect of the KLVFF peptide or KLVFF peptide dendrimer additives on cellular toxicity was observed, results are solely shown for the oligomeric conditions (Figure 5.11). Cell culture medium, **P22'** and **D1P5'** were used as a control. Medium alone and **P22'** alone showed to be not toxic to cells, whereas **D1P5'** resulted in a 50% decrease in cell viability. In addition, oligomers of Aβ(1-42) alone also showed a 50% decrease in cell viability.

Interestingly, the peptide Aβ(1-42) incubated simultaneously with cells and **P22'** showed no toxicity as evaluated using the MTT assay. This indicated that **P22'** inhibited the formation of toxic aggregates of Aβ(1-42). Even more remarkable, incubation of Aβ(1-42) with cells and the dendrimer **D1P5'** (both showed 50% decrease in cellular activity) under oligomeric conditions gave rise to aggregates that showed only a decrease in viability of approximately 10%.

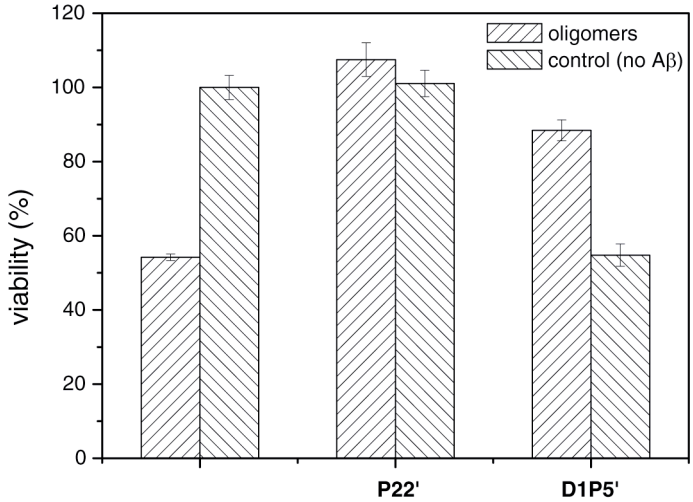


Figure 5.11 *Aβ toxicity as determined by MTT, KLVFF peptide **P22'** and KLVFF-dendrimer **D1P5'** prevent Aβ toxicity.*

In summary, the aggregation behavior of oligomeric Aβ(1-42) was affected *in vitro* by the addition both the KLVFF-peptide and the KLVFF peptide dendrimer. In case of peptide dendrimer addition to Aβ(1-42), longer and thicker fibrillar aggregates were observed,

indicating the presence of a dendritic effect. Addition of either peptide or peptide dendrimer to Aβ(1-42) reduced overall toxicity.

5.6. CONCLUSIONS

Several peptide dendrimers were synthesized with the aim of studying their (multivalent) behavior *in vitro* and *in vivo*. This preliminary investigation was done to have a first impression of the use of peptide dendrimers in biomedical applications.

Preliminary studies *in vivo* showed that the vaccination of rats or rabbits with peptide dendrimers did not result in an immune response. Derivatives of GnRH were attached to dendrimers and tested for their immune response in rats. No antibodies against GnRH peptides were found in the sera of the rats. To investigate if this low immune response was due to the dendrimer structure or due to the combination of the peptide sequence with the dendrimer, another *in vivo* system was evaluated. Dendrimers with the NPNA motif against malaria were tested for their immune response in rabbits. Peptide dendrimers that were not coupled to KLH showed no response, whereas the KLH-coupled constructs all showed an immune response. As the peptide itself was still biologically active, the low immune response was most likely due to the dendrimer.

Preliminary *in vitro* studies were more promising. A multivalent RGD-containing dendrimer seemed to be more effective in inhibiting the binding of a biotinylated RGD peptide to an integrin-coated surface than its monovalent counterpart. This indicated a possible multivalent effect of the dendrimer.

The most promising results were obtained when investigating the influence of a peptide dendrimer on Aβ aggregation and toxicity. A clear effect was seen on aggregation behavior; under oligomer-forming conditions of Aβ, large fibrillar structures were obtained that showed different morphology than the fibrils of Aβ alone. In addition, the aggregates showed a significant decrease in toxicity, indicative of a protective function of the peptide dendrimer towards Aβ oligomers.

An initial step was made towards the use of peptide dendrimers *in vitro* and *in vivo*. The methodology of attaching peptides to dendrimers via native chemical ligation is a very interesting one and can be used to synthesize a variety of peptide dendrimers. The use of the resulting peptide dendrimers in biomedical applications is still an unresolved issue. Peptide dendrimers used in this research have not shown to be useful as synthetic vaccines. Other multivalent structures (more hydrophobic and less flexible) might be more promising in inducing an immune response. However, for drug delivery applications this type of dendrimer is still of high interest. Because of the difficulties observed in *in vivo* studies, it is recommended to first investigate the additive value of peptide dendrimers *in vitro* in more detail before making the step to *in vivo* experiments. It is also advised to conduct further characterization studies to confirm conformation and surface availability of the peptides.

5.7. EXPERIMENTAL SECTION

General procedure for the synthesis of thioester-peptides
See Chapter 3.

pEHWSYGLRPG-MPAL ***P11***
After the coupling of MPAL to the resin, subsequently, Boc-Gly and Boc-Pro were coupled for 10 minutes each; Boc-Arg(Tosyl) was coupled for 20 minutes; Boc-Leu, Boc-Gly and Boc-Tyr(2-Br-Z) were coupled for 10 minutes; Boc-Ser(Bzl) was coupled for 20 minutes; Boc-Trp(For) and Boc-His(DNP) were coupled for 10 minutes. After coupling of Gln, the resin was first washed thoroughly with dichloromethane, before washing with DMF. Finally, Boc-Pyr was coupled for 10 minutes. The resin was washed and dried and the peptide was cleaved from the resin using HF cleavage. The peptide was purified using RP-HPLC using a gradient of 25-45% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 54% yield (95 mg). ESI-MS: found mass: 1549.5 Da; calculated mass: 1549.6 Da.

pEHWSYGLRPGQHWSYGLRPG-MPAL ***P12***
The synthesis of the peptide was done as described above. The coupling of Boc-Gln was coupled for 10 minutes and washed subsequently with DMF, DCM, TFA, DCM, DMF. After completion of the sequence, the resin was washed and dried and the peptide sequence was cleaved from the resin using the above-mentioned HF cleavage procedure. The peptide was purified using RP-HPLC using a gradient of 30-50% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 36% yield (41 mg). ESI-MS: found mass: 2897.4 Da; calculated mass: 2898.0 Da.

pEHWSYkLRPGQHWSYkLRPG-MPAL ***P13***
The synthesis of the peptide was done as described above. The coupling of Boc-D-Lys(2-Cl-Z) was coupled for 10 minutes and washed as usual. After completion of the sequence, the resin was washed and dried and the peptide sequence was cleaved from the resin using the above-mentioned HF cleavage procedure. The peptide was purified using RP-HPLC using a gradient of 25-45% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 30-46% yield (27-49 mg). ESI-MS: found mass: 3040.0 Da; calculated mass: 3040.3 Da.

[pEHWSYGLRPGC]$_4$-dendrimer ***D1P11***
[C]$_4$-dendrimer (5.1 mg, 6.9×10^{-3} mmol, 1 eq) was dissolved in 1 mL of Tris buffer pH 8.0 containing 6 M guanidine. The solution was added to pEHWSYGLRPG-MPAL (54.2 mg, 3.4×10^{-2} mmol, 5 eq). Thiophenol and benzylmercaptan (2 v% each) were added and the pH was checked (7). The mixture was vigorously stirred and reacted at 37 °C for 1 hour. The peptide dendrimer was purified using RP-HPLC using a gradient of 25-45% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure product was obtained in 21% yield (7.8 mg). ESI-MS: found mass: 5390.3 Da; calculated mass: 5390.2 Da.

[pEHWSYGLRPGQHWSYGLRPGC]$_4$-dendrimer ***D1P12***
[C]$_4$-dendrimer (1.8 mg, 2.5×10^{-3} mmol, 1 eq) was dissolved in 2 mL of Tris buffer pH 8.0 containing 6 M guanidine. The solution was added to pEHWSYGLRPGQHWSYGLRPG-MPAL (27.7 mg, 9.5×10^{-3} mmol, 4 eq). Thiophenol and benzylmercaptan (2 v% each, 40 µL) were added and the pH was checked (7.5). The mixture was vigorously stirred and reacted at 37 °C for 1.5 hour. The peptide dendrimer was purified using dialysis in water. After lyophilization, the pure product was obtained in 44 % yield (11 mg). ESI-MS: found mass: 10116.9 Da; calculated mass: 10119.4 Da.

[pEHWSYkLRPGQHWSYkLRPGC]$_4$-dendrimer ***D1P13***
[C]$_4$-dendrimer (1.6 mg, 2.2×10^{-3} mmol, 1 eq) was dissolved in 1.5 mL Tris buffer pH 8.0 containing 6 M guanidine. The solution was added to pEHWSYkLRPGQHWSYkLRPG-MPAL (27 mg, 8.9×10^{-3}

mmol, 4 eq). Thiophenol and benzylmercaptan (2 v% each, 30 μL) were added and the pH was checked (7.5). The mixture was vigorously stirred and reacted at 37 °C for 1.5 hour. The peptide dendrimer was purified using dialysis in water. After lyophilization, the pure product was obtained in 38 % yield (9 mg). ESI-MS: found mass: 10689.0 Da; calculated mass: 10688.4 Da.

[pEHWSYkLRPGQHWSYkLRPGC]$_8$-dendrimer ***D2P13***

[C]$_4$-dendrimer (2.6 mg, 1.6×10^{-3} mmol, 1 eq) was dissolved in 1.5 mL of Tris buffer pH 8.0 containing 6 M guanidine. The solution was added to pEHWSYkLRPGQHWSYkLRPG-MPAL (40 mg, 1.3×10^{-2} mmol, 8 eq). Thiophenol and benzylmercaptan (2 v% each, 30 μL) were added and the pH was checked (7). The mixture was vigorously stirred and reacted at 37 °C for 2 hours. The peptide dendrimer was purified using dialysis in water. After lyophilization, the product couldn't be analyzed.

Elisa test GnRH-dendrimers

96-wells plates were coated with 100 μL of a 0.2% solution of glutardialdehyde (GDA) in 0.1 M sodium phosphate buffer of pH 5.0. After incubation for 4 hours at room temperature, the plates are slapped empty and washed three times with a 0.1 M sodium phosphate buffer of pH 8.0. Then, 100 μL of a 1 mg/mL solution of GnRH-dendrimers in a 0.1 M sodium phosphate buffer of pH 8.0 was added into each well. After incubation for 3 hours at 37C, the plates were emptied to dryness and washed three times with a 0.1 M sodium phosphate buffer of pH 8.0. The plates were subsequently incubated for 1 hour at 37 °C with several dilutions of sera; 1/10, 1/30, 1/100, 1/300, 1/1000, 1/3000, 1/10000, and 1/30000 (samples were diluted with PEM, Peptide ELISA Medium; 4% horse-serum in PBS). The plates were washed 3 times with a mixture of PBS and Tween. The plates were incubated with GAR-PO (goat-anti-rat-HRP, Southern Biotech, Cat. No. 3010-5, Ig(H+L)-HRP, mouse-absorbed (minmal cross-reaction with mouse-IgG)) for 1 hour at 25 °C. The plates were washed 3 times with a mixture of PBS and Tween. Finally, 2,2′-azido-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was added and after 45 minutes the samples were measured for their optical density at405 nm.

Vaccination of rats

The peptide dendrimer was dissolved in PBS (0.66 mg/mL). Of this solution 300 μL was added to 100 μL of adjuvant. The adjuvant used in this research is CoVaccine and was kindly donated by CoVaccine BV, Utrecht, The Netherlands. The resulting solution (400 μL) was used to vaccinate the rats: 100 μL was injected intramuscularly in the left hind leg and 100 μL in the right hind leg; 100 μL was injected subcutaneously in the left flank and 100 μL in the right flank. Vaccination was done on day 0, day 28, and day 56. Blood samples were taken at 4, 6, 8, and 10 weeks. After 12 weeks the rats were bled.

AcGANPNAAGG-MPAL ***P14***

After the synthesis of MPAL to the resin, subsequently, Boc-Gly, Boc-Gly, Boc-Ala and Boc-Ala were coupled for 10 minutes each, Boc-Asn(xantyl) was coupled for 20 minutes, Boc-Pro was coupled for 10 minutes, Boc-Asn(xantyl) was coupled for 20 minutes, Boc-Ala, and Boc-Gly were coupled for 10 minutes. After deprotection ot the Boc groups of the final glycine residue, the N-terminus was acetylated by adding a mixture of 0.5 M acetic anhydride and 0.5 M piperidine in DMF for 2×2 minutes. The resin was washed and dried and the peptide sequence was cleaved from the resin using the above-mentioned HF cleavage procedure. The peptide was purified using RP-HPLC using a gradient of 5-25% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 43% yield (50.6 mg). ESI-MS: found mass (*m/z*): 970.3 Da; calculated mass: 970.0 Da.

AcCANPNAA-MPAL ***P15***

After deprotection of the final cysteine residue, the N-terminus was acetylated by adding a mixture of 0.5 M acetic anhydride and 0.5 M piperidine in DMF for 2×2 minutes. The resin was washed and dried and the peptide was cleaved from the resin by HF cleavage. The peptide was purified using RP-HPLC using a gradient of 10-30% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 75% yield (30 mg). ESI-MS: found mass (*m/z*): 902.4 Da; calculated mass: 902.0 Da.

CANPNAA-MPAL ***P16***
After the synthesis of MPAL to the resin, subsequently, Boc-Ala and Boc-Ala were coupled for 10 minutes each, Boc-Asn(xantyl) was coupled for 20 minutes, Boc-Pro was coupled for 10 minutes, Boc-Asn(xantyl) was coupled for 20 minutes, Boc-Ala, and Boc-Cys(4-Me-Bzl) were coupled for 10 minutes each. The resin was washed and dried and the peptide sequence was cleaved using the HF cleavage. The peptide was purified using RP-HPLC using a gradient of 10-30% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 47% yield (56.1 mg). ESI-MS: found mass (*m/z*): 860.2 Da; calculated mass: 860.0 Da.

clickCANPNAA-MPAL ***P6***
See Chapter 3.

cyclic(CANPNAA) ***P17***
The peptide CANPNAA-MPAL (45.9 mg, 5.3×10^{-2} mmol) was dissolved in 4.6 ml of a Tris buffer pH 8.0 containing 6 M guanidine. Thiophenol (2 v%, 92 μL) and benzylmercaptan (2 v%, 92 μL) were added, the pH was checked (7.5) and the mixture was vigorously stirred. After 1 hour of reaction time, the peptide dendrimer was purified using RP-HPLC using a gradient of 10-30% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 76% yield (26.2 mg). ESI-MS: found mass (*m/z*): 642.4 Da; calculated mass: 641.7 Da.

[AcGANPNAAGGC]$_4$-dendrimer ***D1P14***
[C]$_4$-dendrimer (4 mg, 5.4×10^{-3} mmol, 1 eq) was dissolved in 1 mL Tris buffer pH 8.0 containing 6 M guanidine and AcGANPNAAGG-MPAL (22.3 mg, 2.2×10^{-2} mmol, 4 eq) was added. Thiophenol (2 v%, 20 μL) and benzylmercaptan (2 v%, 20 μL) are added, the pH is checked (7) and the mixture was vigorously stirred. After 1 hour of reaction time, the peptide dendrimer was purified using RP-HPLC using a gradient of 10-30% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide dendrimer was obtained in 29% yield (6 mg). ESI-MS: found mass: 3735.2 Da; calculated mass: 3736.1 Da.

[AcGANPNAAGGC]$_8$-dendrimer ***D2P14***
[C]$_8$-dendrimer (3.9 mg, 2.4×10^{-3} mmol, 1 eq) was dissolved in 1 mL 6 M guanidine buffer of pH 8.0 and AcGANPNAAGG-MPAL (18.8 mg, 1.9×10^{-2} mmol, 8 eq) was added. Thiophenol (2 v%, 20 μL) and benzylmercaptan (2 v%, 20 μL) are added, the pH is checked (7.5) and the mixture was vigorously stirred. After 1 hour of reaction time, the peptide dendrimer was purified using dialysis in water. The pure peptide dendrimer was obtained in 32% yield (6 mg). ESI-MS: found mass: 7611.3 Da; calculated mass: 7612.4 Da.

[AcCANPNAAC]$_4$-dendrimer ***D1P15***
[C]$_4$-dendrimer (6.1 mg, 8.3×10^{-3} mmol, 1 eq) was dissolved in 1.5 mL Tris buffer pH 8.0 containing 6 M guanidine and AcCANPNAA-MPAL (30 mg, 3.3×10^{-2} mmol, 4 eq) was added. Thiophenol (2 v%, 30 μL) and benzylmercaptan (2 v%, 30 μL) were added, the pH was checked (7.5) and the mixture was vigorously stirred. After 1 hour of reaction time, the peptide dendrimer was purified using RP-HPLC using a gradient of 10-30% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide dendrimer was obtained in 43% yield (12.5 mg). ESI-MS: found mass: 3464.1 Da; calculated mass: 3464.0 Da.

[clickCANPNAAC]$_4$-dendrimer ***D1P6***
See Chapter 3.

[maleimide]$_4$-dendrimer ***DM1***
A 1:3 mixture of THF and a saturated solution of sodium bicarbonate in water was made. The first generation poly(propylene imine) dendrimer (127 mg, 0.4 mmol, 1 eq) was dissolved in this mixture and the reaction was cooled to 0 °C. Methoxycarbonylmaleimide (500 mg, 3.2 mmol, 8 eq) was added in small portions. The reaction mixture was stirred at 0 °C for 4 hours, the pH was checked regularly and extra solvent was added when precipitations were seen. After 4 hours, the product was extracted three times using ethyl acetate, the organic layers were combined, washed subsequently with water and brine and dried over magnesium sulfate. After evaporation under reduced pressure, the product

ws obtained as a yellow powder in 74% yield (188 mg). ^{1}H NMR ($CDCl_3$) d (ppm): 6.7 (s, 8H, 8×CH (maleimide)), 3.5 (t, 8H, 4×CH_2 (next to maleimide)), 2.4 (t, 8H, 4×CH_2N (dendrimer-arm)), 1.8-1.2 (m, 16H, 4×CH2 (dendrimer-arm), 2×CH_2 (dendrimer-interior), 2×CH_2 (dendrimer-interior)). ESI-MS: found mass (m/z): 637.3 Da; calculated mass: 636.7 Da.

[cyclic(CANPNAA)]$_4$-dendrimer ***DM1P17***
[maleimide]$_4$-dendrimer (6.5 mg, 1.0×10^{-2} mmol, 1 eq) was dissolved in 1 mL 0.1 M Tris buffer of pH 6.98 and *cyclic*(CANPNAA) (26.2 mg, 4.0×10^{-2} mmol, 4 eq) was added. The pH was checked (7) and the mixture was vigorously stirred for 1.5 hour at room temperature. The peptide dendrimer was purified using RP-HPLC using a gradient of 10-30% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide dendrimer was obtained in 42% yield (12 mg). ESI-MS: found mass: 3203.0 Da; calculated mass: 3203.5 Da.

Peptide dendrimer-KLH conjugates
MHS was coupled to KLH, creating an activated carrier protein. For that purpose KLH was dissolved in a phosphate buffer of pH 8.0 and a solution of MHS in DMF was added. The mixture was vigorously stirred and reacted at room temperature for 5 minutes. To quench the reaction phosphate buffer of pH 6.0 was added and the resulting solution of the activated KLH carrier protein (KLH*) was used for the coupling to peptide dendrimers. Peptide dendrimers were dissolved in DMF and the resulting solution was slowly added to a solution of KLH*. The solution was incubated for 4 hours at room temperature and subsequently a phosphate buffer of pH 6.9 was added until a final volume of 10 mL was reached (concentration 10 mg/mL).

*Ac-C*ANPNAAC*G-OH* ***CP1*** *and Ac-C*GANPNAAGC*G-OH* ***CP2*** *(**: disulfide bridge)*
These peptides were kindly provided by J. T. Meijer (Radboud University Nijmegen).

Vaccination of rabbits
Vaccination of rabbits was done by A. M. Kaan (Nijmegen Center for Molecular Life Sciences, Radboud University Nijmegen). Per dendrimer, 2 rabbits are sacrificed. 100 µL of the dendrimer-solutions (2 mg/mL in PBS) were mixed with 100 µL of Complete Freunds Adjuvant. Rabbits were vaccinated in the lymph node with this solution (200 µL). A "boost" solution was made consisting of a 1:1 mixture of dendrimers solution (1 mg/mL) and Incomplete Freunds Adjuvant. Boost injections (1 mL) were done after 3, 6, 9, 12, 15, 18 and 21 weeks and blood samples (40 mL) were taken after 5, 8, 11, 14, 17, 20 and 23 weeks. Blood samples are centrifuged for 15 minutes and the supernatant (serum) is stored at -20 °C.

Spot-test to determine the presence of specific antibodies in serum
The spot-test resembles the Western Blotting technique. General: PBST buffer is a mixture of PBS buffer and tween; antibodies used in the spot-test are Swar PO (Swine anti Rabbit-peroxidase conjugate); milk powder is used for blocking; blocking buffer consist of PBST and 5% milk powder; incubation buffer consists of PBST and 2.5% milk powder; nitrocellulose strips are 0.5 cm x 5 cm in size (nitrocellulose also used in Western blotting).
Solutions were prepared of peptide **CPI** and **CP2** in PBS to a final concentration of 0.5 mg/mL. These solutions were diluted 2, 4, 8, 16 and 32 times. Nitrocellulose strips were cut from a sheet. On a strip a dilution series was applied by spotting with 1 µL of each of the solutions in the series, the spot was allowed to dry in the air. For each serum sample two strips were prepared, one with peptide CPI the other with peptide CP2, these strips were handled together in the same vessel.
To each vessel 2 mL of blocking buffer was added, the vessel was agitated for 1 hour at RT. Subsequently, the strips were washed 3 times 5 minutes using PBST, they were kept moist in this washing buffer until the next step.
To each vessel 2 mL of incubation buffer was added, to the buffer 5 µL of serum was added (500 times diluted). The diluted serum was incubated overnight at 4 °C and subsequently washed 3 times 5 minutes with PBST, they were kept moist in this washing buffer until the next step. To the strips 2mL solution of specific anti-body 3000 times diluted in PBST milk (2.5%) was added. Following 1 hour of incubation at room temperature the strips were washed again 3 times 5 minutes using PBST. The washing buffer was removed and the strips were put on a glass plate. A freshly mixed solution of the

reagent mixture for the specific antibody was added to the strips and after 1 minute of incubation excess reagents were removed. The glass plate with the strips was covered with plastic foil and the whole was quickly moved to a dark room. Photographic film was put on top of the glass plate and was developed after a one minute exposure.

CRGDf-MPAL ***P18***

After the coupling of MPAL to the resin, subsequently, Boc-D-Phe, Boc-Asp(OcHxl), and Boc-Gly were coupled for 10 minutes each; Boc-Arg(Tos) was coupled for 20 minutes, and Boc-Cys(4-Me-Bzl) was coupled for 10 minutes. The resin was washed and dried and the peptide sequence was cleaved from the resin using HF. The peptide was purified using RP-HPLC using a gradient of 20-40% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 39-54% yield. ESI-MS: found mass (*m/z*): 797.6 Da; calculated mass: 796.9 Da.

cyclic(RGDfC) ***P19***

The peptide CRGDf-MPAL (35.3 mg, 4.4×10^{-2} mmol) is dissolved in 4 mL of 0.1 M Tris buffer of Ph 8.0 containing 6 M guanidine. Thiophenol (2 v%, 80 μL) and benzylmercaptan (2 v%, 80 μL) are added and the pH was checked (7.5). The reaction was vigorously stirred and put at 37 °C. After 1 hour, the cyclic peptide was purified using RP-HPLC using a gradient of 5-25% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 27-39% yield. ESI-MS: found mass (*m/z*): 579.3 Da; calculated mass: 578.6 Da.

cyclic(RGDfC)-biotin ***P20***

The peptide *cyclic*(RGDfC) (21.5 mg, 3.7×10^{-2} mmol, 1 eq) was dissolved in 3 mL 0.1 M Tris buffer of pH 7. Maleimide-biotin (16.8 mg, 3.7×10^{-2} mmol, 1 eq) was added, and the mixture was vigorously stirred for 1 hour at room temperature. The peptide was purified using RP-HPLC using a gradient of 10-30% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 13% yield. ESI-MS: found mass (*m/z*): 1029.4 Da; calculated mass: 1030.1 Da.

maleimide-GGG-MPAL ***M8***

After the coupling of MPAL to the resin, 3 × Boc-Gly were coupled for 10 minutes. Maleimide-β-Ala was coupled in the same way as the normal amino acids are coupled, using HBTU and DIPEA in DMF for 10 minutes. The resin was washed and dried and the peptide sequence was cleaved from the resin using HF. The peptide (104.7 mg) was not purified before use. ^{1}H NMR (DMSO-*d6*): δ (ppm): 8.5 (t, 1H, NH), 8.3 (t, 1H, NH), 8.2 (t, 1H, NH), 7.95 (d, 1H, NH), 7.3 (s, 1H, NH_2), 6.9 (s, 1H, NH_2), 4.2 (q, 1H, CH), 4.0 (d, 2H, CH_2), 3.7 (d, 2H, CH_2), 3.65 (d, 2H, CH_2), 3.61 (d, 2H, CH_2), 3.6 (d, 2H, CH_2), 3.0 (t, 2H, CH_2), 2.4 (t, 2H, CH_2), 1.55 (m, 1H, CH), 1.4 (m, 2H, CH_2), 0.95 (d, 3H, CH_3), 0.9 (d, 3H, CH_3). ^{13}C NMR (DMSO): δ (ppm): 199.2 (CO), 175.4 (CO), 172.0 (2×CO), 171.2 (CO), 171.0 (CO), 170.8 (CO), 170.4 (CO), 135.8 (2×CH), 52.0 (CH), 49.9 (CH_2), 43.3 (CH_2), 43.0 (CH_2), 42.1 (CH_2), 35.8 (CH_2), 35.0 (CH_2), 34.8 (CH_2), 25.5 (CH_2), 24.9 (CH), 24.2 (CH_3), 22.8 (CH_3). ESI-MS: found mass (*m/z*): 541.3 Da; calculated mass: 540.5 Da.

cyclic(RGDfC)-MPAL ***P21***

The peptide *cyclic*(RGDfC) (10.1 mg, 1.7×10^{-2} mmol, 1 eq) was dissolved in 4 mL 0.1 M Tris buffer of pH 6.98. Maleimide-GGG-MPAL (15 mg, 2.7×10^{-2} mmol, 1.5 eq) was added and the mixture was reacted for 2 hours and subsequently lyophilized. The peptide was purified using RP-HPLC using a gradient of 5-25% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA) during 90 minutes. The pure peptide was obtained in 77% yield (25.3 mg). ESI-MS: found mass (*m/z*): 1118.6 Da; calculated mass: 1119.2 Da.

[cyclic(RGDfC)]$_4$-dendrimer ***D1P21***

Cyclic(RGDfC)-MPAL (20 mg, 1.7×10^{-2} mmol, 4 eq) is dissolved in 1.5 mL 6 M guanidine buffer of pH 8.0 and [C]$_4$-dendrimer (3.3 mg, 4.4×10^{-3} mmol, 1 eq) was added. Thiophenol (2 v%, 30 μL) and benzylmercaptan (2 v%, 30 μL) are added, the pH is checked (7) and the mixture was vigorously stirred. After 1 hour of reaction time, the peptide dendrimer was purified using RP-HPLC using a gradient of 20-40% of buffer B (1:9 water:acetonitrile + 0.1% TFA) in buffer A (water + 0.1% TFA)

during 90 minutes. The pure peptide was obtained in 28% yield (5.5 mg). ESI-MS: found mass: 4332.5 Da; calculated mass: 4332.8 Da.

ELISA

Buffer A: 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 20 mM Tris at pH 7.4. Buffer B: 5% (w/v) milk powder, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 50 mM Tris at pH 7.4. Polystyrene wells are irradiated resulting in acid groups at the surface. The resulting high binding plate was coated with integrin $\alpha_V\beta_3$ by addition of 45 µL of buffer A and 5 µL of a 100 µg/mL-solution of integrin $\alpha_V\beta_3$ (Chemical International) in buffer A (0.5 µg per well). The plates were incubated overnight at 4 ^oC. The plates were emptied to dryness, 100 µL of buffer B was added and the plates were incubated for 2 hours at room temperature. The plates were emptied to dryness and washed with TBS and 0.1% tween. Concentrations of **P12** were made in buffer B in flexiplates. From these solutions 50 µL is added to each well until a final concentration of 1000, 100, 10, 1, 0.1 and 0.01 nM per well was reached and the plates were incubated for 3 hours at room temperature. In the competitive ELISA the concentration of **P12** was 100 nM in each measurement, whereas the final concentrations of **P11** and **D1P13** were 1000, 100, 10, 1 and 0.1 nM. After incubation, the plates were emptied to dryness and washed with TBS and 0.1% tween. HRP-antibody was diluted 2000 times and per well 50 µM of the resulting solution was added and the plates were incubated for 1 hour at room temperature. The plates were emptied to dryness and washed with TBS and 0.1% tween. A 1:1 solution of TMB / UP was made and per well 50 µL was added. After 30 minutes the reaction was quenched with 50 µL sulfuric acid per well and the absorption at 450 nm was measured using a plate reader on a Multiscan Ascent Thermo Electron Corporation apparatus.

Determination of K_d

The dissociation constant K_d is the equilibrium constant between the concentration of unbound integrin and RGD in the well and the concentration of integrin–RGD complex (equation 1).

$$[\text{integrin}] + [\text{RGD}] \underset{K_d}{\overset{K_a}{\rightleftharpoons}} [\text{complex}]$$

$$K_d = \frac{[\text{integrin}]\,[\text{RGD}]}{[\text{complex}]} \qquad \text{from which:} \qquad [\text{complex}] = \frac{[\text{integrin}]\,[\text{RGD}]}{K_d} \tag{1}$$

As the concentration of bound integrin equals the total concentration of integrin minus the concentration of integrin-RGD complex, equation 1 can be rewritten to:

$$[\text{integrin}] = [\text{integrin}]_{\text{total}} - [\text{complex}]$$

$$[\text{complex}] = \frac{([\text{integrin}]_{\text{total}} - [\text{complex}])\,[\text{RGD}]}{K_d} = \frac{[\text{integrin}]_{\text{total}}\,[\text{RGD}]}{K_d} - \frac{[\text{complex}]\,[\text{RGD}]}{K_d} \tag{2}$$

In turn equation 2 can be rewritten to:

$$[\text{complex}] + \frac{[\text{complex}]\,[\text{RGD}]}{K_d} = [\text{complex}]\,\frac{K_d + [\text{RGD}]}{K_d} = \frac{[\text{integrin}]_{\text{total}}\,[\text{RGD}]}{K_d} \tag{3}$$

The optical density is a measure for the concentration of integrin-RGD complex formed and thus, equals the total concentration of integrin times the concentration of RGD, divided by the dissociation constant plus the concentration of RGD. Here, the assumption is made that the concentration of unbound RGD equals the total RGD concentration as the RGD peptide is added in large excess.

$$\text{OD} \sim [\text{complex}] = \frac{[\text{integrin}]_{\text{total}}\,[\text{RGD}]}{K_d + [\text{RGD}]} \qquad \text{in which:} \qquad [\text{RGD}] \cong [\text{RGD}]_{\text{total}} \tag{4}$$

Equation 4 was used to fit the data of the measurement and calculate the dissociation constant K_d.

*KLVFFGG-MPAL **P5'***

See Chapter 3.

*$[KLVFFGGC]_4$-dendrimer **D1P5'***

See Chapter 3.

*AcKLVFF-C6-click **P22** and KLVFF-C6-click **P22'***

These peptides were kindly provided by dr. J.G. Linhardt.

Cell Culture

Cell culture reagents were purchased from Gibco BRL (Gaithersburg, MD). SK-N-SH human neuroblastoma cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μg/mL streptomycin and 300 μg/mL glutamine. To induce neuronal differentiation, the SK-N-SH cells were cultured for 5 days in the presence of 10 μM all-trans retinoic acid.

Peptide solubilization and aggregation

The Aβ(1-42) peptide was initially dissolved in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) (1 mg/mL) and separated into aliquots in sterile microcentrifuge tubes. HFIP was removed under vacuum in a Speedvac, and the peptide film was stored desiccated at -20 °C. For the aggregation protocols, the peptide was first resuspended in dry dimethylsulfoxide (DMSO) to a concentration of 2.5 mM. Then for oligomeric conditions, phenol red-free DMEM (Gibco) was added to bring the peptide to a final concentration of 100 μM and incubated at 4 °C for 24 h. For fibrillar conditions, 10 mM HCl was added to bring the peptide to a final concentration of 100 μM and incubated for 24 h at 37 °C. Characterization of the Aβ aggregates was performed by electron microscopy and Thioflavin T fluorescence. The peptide KLVFF and corresponding $[KLVFFGGC]_4$-dendrimer is dissolved in HFIP and added to the Aβ peptide in different ratios.

Electron Microscopy

For electron microscopy, 5 μL of the sample were adsorbed onto 300-mesh formvar-coated grids for 5 min, subsequently excess fluid was filtered off, the samples were negatively stained with 1% uranyl acetate for 5 min and viewed with a Philips (FEI) EM420 transmission electron microscope equipped with a SIS MegaviewII ccd camera for image acquisition.

Thioflavin T Fluorescence Assay

In the thioflavine assay, thioflavine T (Thio T) binds specifically to amyloid fibrils. Binding of the dye to amyloid fibrils in suspension generates a new excitation (ex) (absorption) maximum at 450 nm and enhanced emission (em) at 492 nm, as opposed to the 385 nm (ex) and 445 nm (em) of the free dye. This change is dependent on the aggregated state, as monomeric or dimeric peptides do not induce this change. In this fluorometric assay the amyloid fibril assembly process can be monitored, and the technique is useful for studies of the effects of various substances on fibril formation.

A 100 μM aqueous solution of Thioflavin T (Th T) was prepared and filtered through a 0.2 μm filter. Fluorescence measurements for the 100 μM Aβ incubations were performed in a 96-well microplate (Ex = 450 nm, Em = 482 nm). To each well was added 5 μL of 100 μM Aβ preparations, 10 μL of 100 μM ThT and 85 μL of 90 mM glycine (pH 8.5).

Assay of cell viability

The cytotoxicity of the Aβ aggregates was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were plated on 96-well plates in 100 μL of fresh medium. After 24 hours, the media were changed with 100 μL of fresh media supplemented with 10.0 μM of oligomeric or fibrillar Aβ aggregates. After 48 hours, 100 μL of MTT solution in cell culture medium was added to the cell cultures (0.5 mg/mL final concentration) and samples were incubated at 37 °C for 2 hours. After 2 hours, medium was aspired and 50 μL DMSO was added to each well to solubilize the formazan salt. Absorbance values of blue formazan were determined at 590 nm with an automatic 96 well-plate reader.

5.8. REFERENCES

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APPENDIX 1: AMINO ACIDS

All twenty naturally occurring L-amino acids and their corresponding three-letter code and one-letter code abbreviations are shown in Figure A.1. For clarity reasons, one-letter code abbreviations are used throughout this thesis and the stereochemistry is not shown in the peptide dendrimers described. In all cases L-amino acids are used, unless specifically mentioned otherwise. In that case the one-letter code abbreviations are non-capitalized.

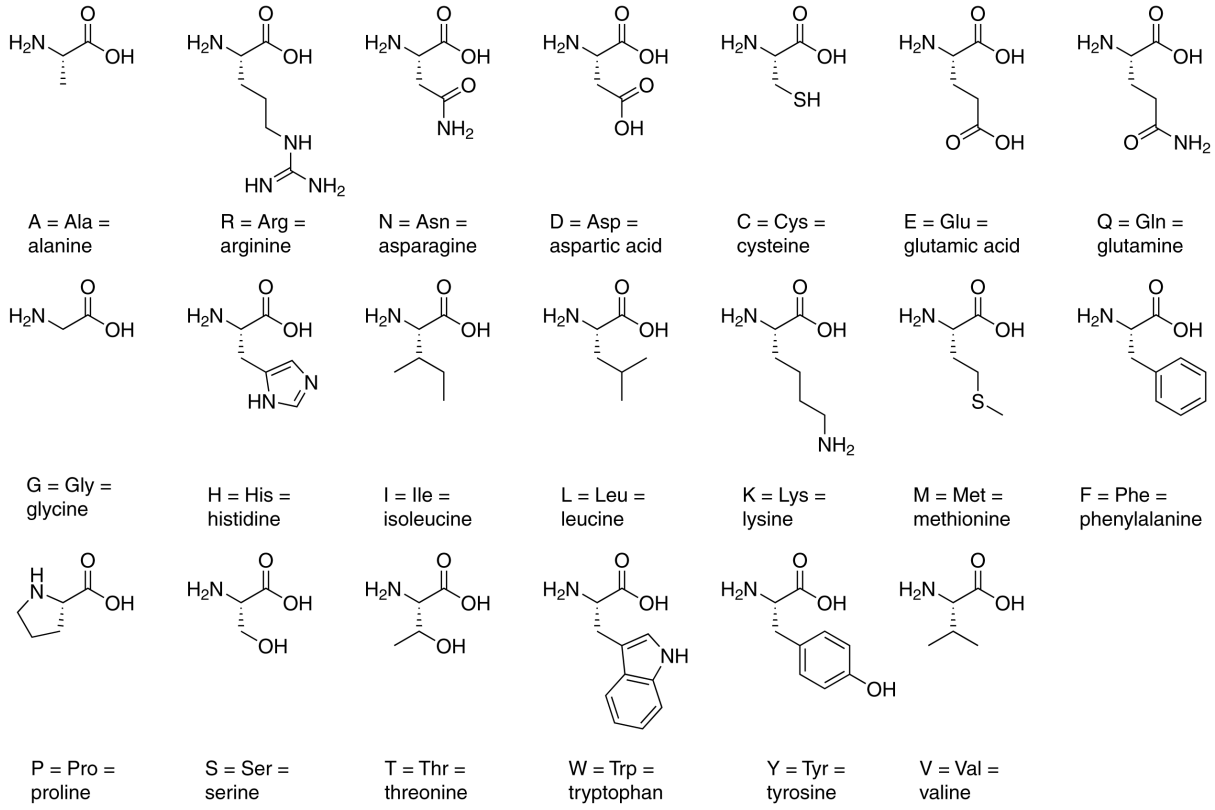

Figure A.1 *All twenty naturally occurring L-amino acids.*

APPENDIX 2: LIST OF ABBREVIATIONS

Aβ	β-amyloid peptide
BME	β-mercaptoethanol
*t*Boc	*tert*-butyloxycarbonyl
CS	circumsporozoite
DNP	3,5-dinitrophenyl
ELISA	enzyme-linked immunosorbent assay
ESI-MS	electrospray ionization mass spectrometry
FSH	follicle stimulating hormone
GnRH	gonadotropin-releasing hormone
KLH	keyhole limpet hemocyanin
LH	luteinizing hormone
MALDI-TOF	matrix assisted laser desorption ionization – time of flight
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PAMAM	poly(amido amine)
PPI	poly(propylene imine)
Pyr, pE	pyroglutamic acid
RP-HPLC	reversed phase high performance liquid chromatography
SPPS	solid phase peptide synthesis
TBE	trypan blue exclusion
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
TMB	3,3′,5,5′-tetramethylbenzidine
Tris	tris(hydroxymethyl)aminomethane hydrochloride

SUMMARY

Dendrimers are highly branched, monodisperse molecular architectures possessing a high number of end groups. Due to their unique structure and properties, dendrimers are considered as ideal candidates for biomedical applications. They adopt a globular shape and are polyvalent, two features that are often seen in naturally occurring systems. In recent years, dendritic scaffolds have been explored for their use in biomedical applications such as drug delivery, synthetic vaccination, magnetic resonance imaging or tissue engineering. These initial results are promising and serve as a foundation for the research described in this thesis. The first target is the development and evaluation of different chemical approaches for the efficient attachment of biological probes onto dendrimers. Once these dendrimers are prepared, the bioactivity *in vitro* and *in vivo* will be studied.

A supramolecular approach for the attachment of drugs to dendrimers is described in Chapter 2. A convenient methodology has been developed for the synthesis of water-soluble dendrimers possessing a hydrophobic domain next to the basic core. The dendrimers are not toxic to rat macrophages and are used as molecular nanocontainers to supramolecular encapsulate small apolar drugs, such as cortisol. As these drugs have low solubility in water, their local concentration is raised when encapsulated in the dendrimer. To strengthen the complex formation of dendrimer and drug, cortisol was modified with a urea-glycine tail in order to have a combination of multiple interactions (electrostatic, hydrophobic and hydrogen bonding) between dendrimer and cortisol. From this cortisol prodrug, the active cortisol is expected to be released by either enzymatic or basic cleavage of the ester bond. The prodrug in water in the presence of the dendrimer showed a more than five-fold increase in solubility. Both cortisol and the cortisol prodrug show similar biological activity when integrated with the dendrimer, indicating the release of active cortisol from the carrier and degradation of the ester bond in case of the prodrug.

To achieve biological modification of dendrimers in a covalent way, a general synthetic method was developed for the conjugation of synthetic thioester-peptides to cysteine-functionalized dendrimers using native chemical ligation (Chapter 3). The first, second and third generation cysteine dendrimers have successfully been prepared and several thioester-peptides were synthesized by solid phase peptide synthesis. Ligation of thioester-peptides to the first generation and second generation cysteine dendrimers succeeded for several thioester-peptides and peptide-dendrimer products were isolated in good yields. The synthesis of third generation peptide dendrimers presented several problems in both the analysis and isolation of the products. Most likely, a heterogeneous mixture is obtained due to steric hindrance at the periphery. The modular approach presented provides access to a wide variety of well-defined multivalent peptides that are attractive for understanding the

fundamental mechanisms of multivalency in biological interactions and for potential use in biomedical applications in targeted drug delivery, molecular imaging and immunology.

The introduction of peptides on cysteine-dendrimers using the native chemical ligation technique results in a pendant thiol group. Chapter 4 describes the analysis and characterization of the nature of the thiol groups and their conversion into disulfide bonds, both intramolecularly and intermolecularly. The presence of thiol groups after ligation onto the dendrimers is also used as a tool to incorporate additional functionalities (at a later stage) through reaction with maleimides. A general strategy is reported for the introduction of multiple MRI labels and fluorescent labels for optical imaging to peptide-dendrimer scaffolds, utilizing highly efficient chemoselective reaction procedures and resulting in well-defined multivalent dendritic MRI contrast agents. Also, oxime chemistry is used in combination with maleimide-thiol reaction to develop a potential dynamic drug delivery system based on peptide-dendrimer scaffolds. The methodology presented in this chapter shows the efficient use of pendant thiol groups for chemical modification of peptide dendrimers.

In Chapter 5 multivalent binding effects of peptide dendrimers to receptors are evaluated *in vitro* and *in vivo* in a first and preliminary study. Preliminary studies *in vivo* showed that the vaccination of peptide dendrimers as synthetic vaccines against malaria or prostate cancer did not result in an immune response. Preliminary *in vitro* studies were more promising. A multivalent RGD-containing dendrimer seemed to be more effective in inhibiting the binding of a biotinylated RGD peptide to an integrin-coated surface compared to its monovalent counterpart, indicative of a possible multivalent binding effect. However, no reliable quantitative comparison can be made, because of large errors in the measurements and difficulties in reproducibility. The most promising *in vitro* results were obtained when investigating the influence of a peptide dendrimer on the aggregation and toxicity of the β-amyloid peptide (Aβ) that plays an important role in Alzheimer's disease. A clear effect was seen on aggregation behavior; under oligomer-forming conditions of Aβ, large fibrillar structures were obtained that showed low β-sheet content. In addition, the aggregates showed a significant decrease in toxicity, indicative of a protective function of the peptide dendrimer towards Aβ oligomers.

In this thesis a first step was made towards the use of peptide dendrimers *in vitro* and *in vivo*. The methodology of attaching peptides to dendrimers via native chemical ligation is highly efficient and can be used to synthesize a variety of peptide dendrimers. The use of the resulting peptide dendrimers in biomedical applications is still an unresolved issue. For use in synthetic vaccination other multivalent structures (more hydrophobic and less flexible) might be more promising in inducing an immune response. However, for drug delivery applications peptide dendrimers are still of high interest. Because of the difficulties observed in *in vivo* studies, it is recommended to first investigate the additive value of peptide dendrimers *in vitro* in more detail before making the step to *in vivo* experiments.

SAMENVATTING

Dendrimeren zijn sterk vertakte, monodisperse moleculaire architecturen met een groot aantal eindgroepen. Door hun unieke structuur en eigenschappen, worden ze beschouwd als ideale kandidaten voor biomedische toepassingen. Ze zijn bolvormig en polyvalent, twee kenmerken die vaak voorkomen in natuurlijke systemen. Recentelijk zijn dendritische structuren onderzocht voor gebruik in biomedische toepassingen zoals medicijnafgifte, synthetische vaccinatie, Magnetic Resonance Imaging (MRI), of weefseltechnologie. Deze eerste resultaten zijn veelbelovend en dienen als basis voor dit proefschrift. Het eerste doel is de ontwikkeling and evaluatie van verschillende chemische benaderingen voor de efficiente aanhechting van biologische stoffen aan dendrimeren. Wanneer deze dendrimeren gemaakt zijn, wordt de bioactiviteit *in vitro* en *in vivo* getest.

Een supramoleculaire aanpak voor de aanhecting van medicijnen aan dendrimeren is beschreven in hoofdstuk 2. Een geschikte methodologie is ontwikkeld voor de synthese van wateroplosbare dendrimeren met een hydrofoob domein naast de basische kern. De dendrimeren zijn niet toxisch en zijn onderzocht voor gebruik als nanocontainers voor de supramoleculaire opsluiting van kleine apolaire medicijnen, zoals cortisol. Door middel van opsluiting van cortisol in het dendrimeer is de locale concentratie van dit medicijn verhoogd. Om het complex tussen dendrimeer en medicijn te versterken is cortisol gemodificeerd met een ureum-glycine staart om meerdere interacties (elektrostatische, hydrofobe, en waterstofbruginteracties) tussen dendrimeren en cortisol te bewerkstelligen. Cortisol kan worden verkregen uit deze cortisolprodrug door middel van enzymatische of basische hydrolyse van de esterbinding. De prodrug in water in aanwezigheid van het dendrimeer resulteert in een meer dan vijfvoudige verhoging in oplosbaarheid. Zowel cortisol als de prodrug hebben vergelijkbare biologische activiteit wanneer ze aan het dendrimeer gecomplexeerd zijn, wat duidt op de afgifte van actief cortisol van de drager en hydrolyse van de esterbinding.

Naast de supramoleculaire modificatie van dendrimeren is ook een algemene synthetische methode ontwikkeld voor de koppeling van synthetische thioesterpeptides aan cysteïnedendrimeren door middel van natieve chemische ligatie (hoofdstuk 3). De eerste, tweede en derde generatie cysteïnedendrimeren zijn vervaardigd en verscheidene thioesterpeptides zijn gemaakt via vaste fase peptidesynthese. Ligatie van thioesterpeptides aan de eerste en tweede generatie cysteïnedendrimeren was succesvol voor verscheidene peptides en de producten konden in een goede opbrengst worden geïsoleerd. Echter de synthese van derde generatie peptidedendrimeren vertoonde verschillende problemen in zowel de analyse als de isolatie van de producten. Door sterische hindering aan de periferie van het dendrimeer is waarschijnlijk een heterogeen mengsel verkregen. De getoonde modulaire aanpak geeft toegang tot een grote variëteit aan goed gedefinieerde multivalente

peptides. Deze multivalente peptides zijn attractief voor het begrijpen van de fundamentele mechanismen van multivalentie in biologische interacties en voor potentieel gebruik in biomedische toepassingen, zoals in medicijnafgifte, moleculaire beeldvorming en immunologie.

De introductie van peptides aan cysteïnedendrimeren door middel van natieve chemische ligatie resulteert in een vrije thiolgroep. Hoofdstuk 4 beschrijft de analyse en karakterisering van de aard van deze thiolgroepen en hun omzetting naar disulfidebruggen, zowel intermoleculair als intramoleculair. De aanwezigheid van thiolgroepen na ligatie is ook gebruikt als een middel om additionele functionaliteiten toe te voegen via een reactie met maleïmides. Door gebruik te maken van deze reactie is er een algemene strategie ontwikkeld voor de introductie van meerdere MRI labels en fluorescente labels aan peptidedendrimeren, resulterend in goed gedefinieerde multivalente dendritische MRI contrastmiddelen voor beeldvorming. Ook is oximechemie gebruikt in combinatie met de maleïmide-thiol reactie om een potentieel dynamisch medicijnafgiftesysteem gebaseerd op peptidedendrimeren te ontwikkelen.

In hoofdstuk 5 zijn multivalente bindingseffecten van peptidedendrimeren aan receptoren onderzocht *in vitro* en *in vivo* in een eerste en voorbereidende studie. De *in vivo* studies waarin peptidedendrimeren als synthetisch vaccin tegen malaria of prostaatkanker gebuikt worden, laten zien dat peptidedendrimeren geen immuunreactie opwekken in respectievelijk konijnen of ratten. De *in vitro* studies geven betere resultaten. Een multivalent RGD-dendrimeer lijkt sterker te binden aan integrine $\alpha_V\beta_3$ in vergelijking tot de monovalente RGD sequentie, wat duidt op eventuele multivalente binding van het dendrimeer aan de receptor. Echter, de experimenten zijn moeilijk te reproduceren. Veelbelovende resultaten zijn verkregen bij het onderzoeken van de invloed van een peptidedendrimeer op de aggregatie en toxiciteit van het β-amyloid eiwit, dat een belangrijke rol speelt in de ziekte van Alzheimer. Een duidelijk effect op de aggregatie was waargenomen; onder oligomeer-vormende condities worden grote fibrillen gevormd, resulterend in een lagere toxiciteit van de aggregaten. Dit duidt op een beschermende functie van het peptidedendrimeer op aggregaten van het β-amyloid eiwit.

In dit proefschrift is een eerste stap gezet richting het gebruik van peptidedendrimeren *in vitro* en *in vivo*. De methologie om peptides aan dendrimeren te hechten via natieve chemische ligatie is veelbelovend en kan worden gebruikt om een variëteit aan peptidedendrimeren te maken. De toegevoegde waarde van de resulterende peptidedendrimeren in biomedische applicaties is nog onduidelijk. Voor gebruik in synthetische vaccinatie zijn waarschijnlijk andere multivalente structuren meer geschikt (meer hydrofoob en minder flexibel). Echter als medicijnafgiftesysteem kunnen peptidedendrimeren van groot belang zijn. Gezien de moeilijkheden in *in vivo* studies wordt het aanbevolen om de toegevoegde waarde van peptidedendrimeren eerst *in vitro* te bestuderen voor de stap naar *in vivo* experimenten wordt gemaakt.

CURRICULUM VITAE HINKE MALDA

Born in the summer of 1978 Hinke spent a happy childhood in the town of Zwolle with her parents and elder brother. She carried her broad range of interests throughout secondary education. Graduating from the Thorbecke College in 1996, her final exams covered almost the entire spectrum ranging from the natural sciences to languages and liberal arts. Yet the sciences prevailed as she entered the University of Groningen for a degree in synthetic organic chemistry. Her scientific tour de force started with an exploration of asymmetric allylic alkylations using dialkylzincs and phosphoramidite ligands in the group of prof.dr. B.L. Feringa. Besides being an avid student, Hinke was actively involved in the student community and presided over the chemical students' association in 1998-1999. An era of devotion as much to the asymmetric alkylations as to the renowned Groninger "life-after-five" came to an end in 2001. Hinke combined her academic aptitude with practical application during a 4 month placement at Novartis Pharma AG in Basel, Switzerland, and a brief interlude working for Syncom BV in Groningen. With a long-standing interest in pharmaceutical chemistry and spurred on by her experiences in Switzerland, she obtained a Ph.D. position in the field of biomedical engineering at the University of Eindhoven under the supervision of prof.dr. E.W. Meijer and dr. M.H.P. van Genderen. The research focused on the design of dendrimers for use in biomedical applications. Throughout her work she has collaborated intensively with dr. T.M. Hackeng at the University of Maastricht. Hinke's passion for travel has taken her to all corners of the world and her research received international acclaim at the 19th symposium of the American Peptide Society in San Diego, USA, as well the International Dendrimer Symposium in Berlin, Germany. When not working in the lab or traveling, Hinke enjoys home-made gourmet food and volleyball.

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