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Espoo 2008

MULTIVALENT DENDRONS FOR HIGH-AFFINITY DNA BINDING

Doctoral Dissertation

Mauri Kostiainen

Helsinki University of Technology
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Department of Engineering Physics

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Dissertation for the degree of Doctor of Science in Technology to be presented with due permission of the Faculty of Information and Natural Sciences for public examination and debate in Auditorium AS1 at Helsinki University of Technology (Espoo, Finland) on the 29th of May, 2008, at 12 noon.

Helsinki University of Technology
Faculty of Information and Natural Sciences
Department of Engineering Physics

Teknillinen korkeakoulu
Informaatio- ja luonnontieteiden tiedekunta
Teknillisen fysiikan laitos

Distribution:
Helsinki University of Technology
Faculty of Information and Natural Sciences
Department of Engineering Physics
P.O. Box 5100
FI - 02015 TKK
FINLAND
URL: http://omm.tkk.fi/
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ISBN 978-951-22-9371-1
ISBN 978-951-22-9372-8 (PDF)
ISSN 1795-2239
ISSN 1795-4584 (PDF)
URL: http://lib.tkk.fi/Diss/2008/isbn9789512293728/

TKK-DISS-2471

Painotalo Casper Oy
Espoo 2008

ABSTRACT OF DOCTORAL DISSERTATION	HELSINKI UNIVERSITY OF TECHNOLOGY P.O. BOX 1000, FI-02015 TKK http://www.tkk.fi

Author Mauri Kostiainen

Name of the dissertation
Multivalent dendrons for high-affinity DNA binding

Manuscript submitted 20.02.2008	Manuscript revised
Date of the defence 29.05.2008	
☐ Monograph	☒ Article dissertation (summary + original articles)

Faculty	Faculty of Information and Natural Sciences
Department	Department of Engineering Physics
Field of research	Molecular Materials
Opponent(s)	Prof. David Haddleton
Supervisor	Prof. Olli Ikkala
Instructor	Prof. Olli Ikkala, Prof. David K. Smith

Abstract

In this Thesis the synthesis of various Newkome-type polyamine dendrons and their DNA binding properties is presented. These cationic dendrons bind DNA with extremely high affinity through multivalent ionic interactions. Dendrons with *o*-nitrobenzyl linked surface groups can be cleaved from the dendron framework by optical irradiation resulting in rapid release of the covalently bound surface groups and non-covalently bound DNA, due to dendron degradation and charge switching multivalency. *N*-maleimido cored dendrons can be attached onto protein surfaces in site-specific manner to yield exactly defined one-to-one protein-polymer conjugates, where the number of dendrons and their attachment site on the protein surface is precisely known. The resulting protein-dendron conjugates bind DNA with high affinity. Further studies in gene transfection, cytotoxicity and self-assembly establish relevance in gene therapy and surface patterning.

This Thesis consists of an overview of the following five publications:

1. Kostiainen M.; Hardy J.; Smith D. *Angew. Chem. Int. Ed.* **2005**, *44*, 2556-2559
2. Hardy J.; Kostiainen M.; Smith D.; Gabrielson N.; Pack D. *Bioconjugate Chem.* **2006**, *17*, 172-178
3. Kostiainen M.; Smith D.; Ikkala O. *Angew. Chem. Int. Ed.* **2007**, *46*, 7600-7604
4. Kostiainen M.; Szilvay G.; Smith D.; Linder M.; Ikkala O. *Angew. Chem. Int. Ed.* **2006**, *45*, 3538-3542.
5. Kostiainen M.; Szilvay G.; Lehtinen J.; Smith D.; Linder M.; Urtti A.; Ikkala O. *ACS Nano* **2007**, *1*, 103-113

Keywords self-assembly, dendrimers, dendron, DNA, protein

ISBN (printed)	978-951-22-9371-1	ISSN (printed)	1795-2239
ISBN (pdf)	978-951-22-9372-8	ISSN (pdf)	1795-4584
Language	English	Number of pages	61 p. + app. 41 p.

Publisher Helsinki University of Technology, Department of Engineering Physics

Print distribution Helsinki University of Technology, Department of Engineering Physics

☒ The dissertation can be read at http://lib.tkk.fi/Diss/2008/isbn9789512293728/

VÄITÖSKIRJAN TIIVISTELMÄ	TEKNILLINEN KORKEAKOULU PL 1000, 02015 TKK http://www.tkk.fi

Tekijä Mauri Kostiainen

Väitöskirjan nimi
DNA:ta sitovat monivalenssiset dendrimeerit

Käsikirjoituksen päivämäärä 20.02.2008	Korjatun käsikirjoituksen päivämäärä

Väitöstilaisuuden ajankohta 29.05.2008

☐ Monografia	☒ Yhdistelmäväitöskirja (yhteenveto + erillisartikkelit)

Tiedekunta	Informaatio- ja luonnontieteiden tiedekunta
Laitos	Teknillisen fysiikan laitos
Tutkimusala	Molekyylimateriaalit
Vastaväittäjä(t)	Prof. David Haddleton
Työn valvoja	Prof. Olli Ikkala
Työn ohjaaja	Prof. Olli Ikkala, Prof. David K. Smith

Tiivistelmä

Tämän tutkielman puitteissa valmistettiin amiinipohjaisia Newkome-tyypin dendroneja, sekä tutkittiin niiden kykyä sitoa ja pakata DNA:ta. Kationiset dendronit sitoutuvat DNA:han erittäin voimakkaasti monivalenssisilla ionisilla vuorovaikutuksilla. Jos DNA:ta sitovat pintaryhmät liitetään dendrimeeriin *o*-nitrobentsyyli ryhmällä, dendrimeeri voidaan hajoittaa valon avulla ja täten vapauttaa DNA. Dendronin ytimeen sijoitettavan *N*-maleimido –ryhmän avulla dendroni voidaan kiinnittää proteiinien pintaan ja täten luoda monodisperssejä konjugaatteja, joissa dendrimeerien lukumäärä ja kiinnityskohta on tarkasti määritelty. Proteiini-dendroni -konjugaattien havaittiin sitoutuvan DNA:han erittäin voimakkaasti. Lisäksi konjugaatit eivät ole myrkyllisiä ja kykenevät transfektoimaan DNA:ta soluihin.

Väitöskirja koostuu seuraavista viidestä julkaisusta:

1. Kostiainen M.; Hardy J.; Smith D. *Angew. Chem. Int. Ed.* **2005**, *44*, 2556-2559
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Asiasanat itsejärjestyminen, dendrimeeri, dendroni, DNA, proteiini

ISBN (painettu) 978-951-22-9371-1	ISSN (painettu) 1795-2239
ISBN (pdf) 978-951-22-9372-8	ISSN (pdf) 1795-4584
Kieli englanti	Sivumäärä 61 s. + liit. 41 s.

Julkaisija Teknillinen korkeakoulu, Teknillisen fysiikan laitos

Painetun väitöskirjan jakelu Teknillinen korkeakoulu, Teknillisen fysiikan laitos

☒ Luettavissa verkossa osoitteessa http://lib.tkk.fi/Diss/2008/isbn9789512293728/

PREFACE

My first contact with experimental science was a laboratory trainee period at the Institute of Biotechnology at University of Helsinki in summer 1997 in the laboratory of Harri Savilahti. Since this enthusiastic summer I have been keenly interested in natural sciences, later especially those topics covering the fields of bioorganic and supramolecular chemistry. This interest of mine formed the basis for University and graduate studies, short research projects in various laboratories and research centres and now to the outcome of this Thesis. There are many people who have contributed in one way or another to this work and I wish to thank all of you for your efforts or for just being there for me and making this Thesis possible.

This work is a result of group work and has been carried out mainly in two places. The work was initiated in the Bioinorganic and Supramolecular Chemistry Group of the Department of Chemistry at the University of York and finalised in the Centre of Excellence of the Academy of Finland ("Bio- and Nanopolymer Research group", 77317) at the laboratory of Optics and Molecular materials of the Department of Engineering Physics at the Helsinki University of Technology. I would like to thank my supervisors Prof. David K. Smith and Prof. Olli Ikkala for all of the encouragement, insight and thoughts. It now seems possible for me to see further and understand complex connections with less effort, and this is possible only if one stands on the shoulders of giants.

I am indebted to all those talented people who have given advice, encouragement and kept up the mood during the long hours in the lab, especially John, Jari, Emmi, Sirkku, Géza, Katri and Teija. I would also wish to thank the other members of the research groups and staff for being good friends in and outside laboratory. It has been a privilege to work with you. I would furthermore like to express my gratitude to our important collaborators: Markus B. Linder in VTT Biotechnology, Arto Urtti in University of Helsinki and Daniel W. Pack in University of Illinois at Urbana Champaign.

Financial support from the Finnish Academy's Center of Excellence (Bio- and Nanopolymers research group 77317), Finnish Funding Agency for Technology and Innovation, the National Graduate School in Nanoscience, Finnish Chemical Society, Research Foundation of Orion Corporation, Technological Foundation, Magnus Ehrnrooth Foundation and Emil Aaltonen Foundation is gratefully acknowledged.

Finally, I would like to thank my family and friends for support over the years as well as enduring those long discussions about chemistry – they were not in vain.

Kiitos Elina ja Malla!

Espoo, 20.2.2008

Mauri Kostiainen

LIST OF PUBLICATIONS

I **Kostiainen, M. A.**; Hardy, J. G.; Smith, D. K. *High-Affinity Multivalent DNA Binding by using Low-Molecular Weight Dendrons.* Angewandte Chemie International Edition, **2005**, *44*, 2556-2559

II Hardy, J. G.; **Kostiainen, M. A.**; Smith, D. K.; Gabrielson, N. P.; Pack, D.W. *Dendrons with Spermine Surface Groups as Potential Building Blocks for Nonviral Vectors in Gene Therapy.* Bioconjugate Chemistry, **2006**, *17*, 172-178

III **Kostiainen, M. A.**; Smith, D. K.; Ikkala, O. *Optically Triggered Release of DNA from Multivalent Dendrons by Degrading and Charge-Switching Multivalency.* Angewandte Chemie International Edition, **2007**, *46*, 7600-7604

IV **Kostiainen, M. A.**; Szilvay, G. R.; Smith, D. K.; Linder, M. B.; Ikkala, O. *Multivalent Dendrons for High-Affinity Adhesion of Proteins to DNA.* Angewandte Chemie International Edition, **2006**, *45*, 3538-3542

V **Kostiainen, M. A.**; Szilvay, G. R.; Lehtinen, J.; Smith, D. K.; Linder, M. B.; Urtti, A.; Ikkala, O. *Precisely Defined Protein-Polymer Conjugates: Construction of Synthetic DNA Binding Domains to proteins by Using Multivalent Dendrons.* ACS Nano, **2007**, *1*, 103-113

Through the Thesis, above-mentioned articles will be referred by their Roman numerals.

AUTHOR'S CONTRIBUTION

The research reported in this Thesis is the result of multidisciplinary group work carried out during years 2003 – 2007, mainly in the laboratory of Bioinorganic and Supramolecular Chemistry Group at University of York and the laboratory Optics and Molecular Materials at Helsinki University of Technology.

The author has taken active part in all stages of the design, realisation, analysis, and reporting of the work presented in this Thesis. The author has written the first versions of Publications III – V and a research report which later formed the basis for publication I. In these articles the author is responsible for all experimental work, excluding preparation of **G0** analogue, which was synthesised by John Hardy at the University of York and preparation of Ncys-HFBI and AFM imaging preformed by Géza Szilvay at VTT Biotechnology. In article II the author is responsible for the synthesis of **G1** and **G2**.

The author has also presented the results covered in this Thesis at several international conferences.

CONTENTS

1. BACKGROUND

"We know nothing in reality, for truth lies in an abyss."

- Democritus (c. 420 BC) -

Biologically programmed molecular recognition forms the basis for all complex natural systems. Biology has therefore stimulated the use of self-assembly[a] ("*bottom-up*") approaches for the development of diverse biomimetic nanostructures,[1] which are held together by competing attractive and repulsive forces within a molecular system. DNA is the nature's very own predominant biopolymer for duplication and storage of genetic information in biology, and makes a fascinating building block for self-assembled structures and biotechnological research.[2] DNA can be used even in applications that are not immediately obvious, such as for building highly monodisperse nanostructures[3, 4] and DNA based computing.[5-7] Compounds that bind DNA with high affinity are particularly interesting for protecting DNA and ultimately delivering genetic material into cells; a technique known as gene therapy.[8] However, a single monovalent[b] binding unit can not efficiently bind DNA under physiological conditions and to achieve high-affinity binding multivalent ligands must be utilised. Dendrimers and dendrons are particularly interesting binding agents because of their precisely defined branched structure with a high density of functional surface groups, which can offer multiple simultaneous interactions leading to enhanced binding – the principle known as multivalency[c].[9-12] Furthermore, dendrimers can be designed in such a way that allows their self-assembly through supramolecular interactions.[13-15] Well-defined structures that are held together by non-covalent interactions can be constructed principally in three ways from dendritic building blocks (Figure 1):[13]

1. Using templated or untemplated assembly through a functionalised focal point.
2. Employing noncovalent intermolecular dendron–dendron interactions can give rise to the hierarchical assembly of nanostructured materials.
3. Dendron periphery with multiple surface groups can be functionalised in a desired manner to invoke self-assembly and multivalent binding.[16]

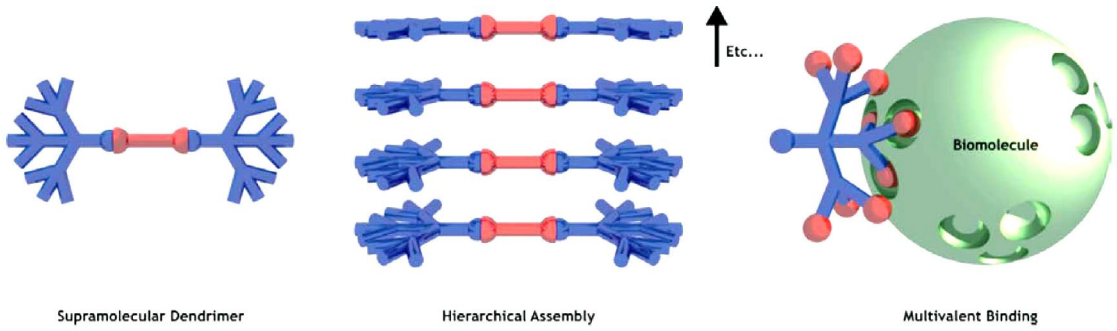

Figure 1. Schematic illustration how dendron based structures are held together by non-covalent interactions.[13]

[a] Term *self-assembly* is defined here as the spontaneous and reversible organization of molecular units into ordered structures by non-covalent interactions.

[b] The International Union of Pure and Applied Chemistry (IUPAC) defines valence as: "*the maximum number of univalent atoms that may combine with an atom of the element under consideration, or with a fragment, or for which an atom of this element can be substituted.*"

[c] In this Thesis term *multivalent binding* is used to describe the binding of two or more entities through separate simultaneous interaction, between multiple (two or more) complementary ligand-receptor functionalities on these entities, resulting in unique thermodynamic features termed as *multivalency*.

1.1. Gene Therapy

Gene therapy is defined as the use of genetic material to alleviate the symptoms of a disease.[8, 17] The principle is very simple: putting therapeutic gene into cells treats the disease. Prospects of successful gene delivery range from slowing the growth of tumours[18-22] and progression of neurodegenerative diseases, such as Alzheimer's and Creutzfeldt-Jakob disease,[23] to alleviating genetic diseases characterised by a single mutation at a defined position on the genome, for example muscular dystrophy[24] and cystic fibrosis [25-27]. However, several hurdles must be overcome before gene therapy can be utilised to treat patients routinely. The key problems in gene therapy are: most importantly, the lack of efficient delivery systems that could navigate DNA through cell membranes and a series of extra- and intracellular barriers, lack of targeting to specific tissue types, lack of long-term effects and strong immune response. The search for an efficient delivery system, which could fulfil all the criteria for successful gene therapy, has now been going on for several decades. The first clinical trial for gene therapy was carried out in 1990 to treat severe combined immunodeficiency[28] and since that several hundred clinical trials have been pulled through. However, there is still no breakthrough to a success story and the discovery of an ideal vector remains as a future challenge.

Nucleic acid used in gene therapy can be either double stranded DNA constructs or single stranded systems, such as antisense oligonucleotides or short interfering RNA (siRNA). Delivery of DNA with a gene coding for particular product commonly results in the increased production of a therapeutic protein, whereas delivery of antisense constructs will usually lead to a reduction of target activity. Although both of these approaches have been extensively studied and the antisense approach has been regarded highly promising, the following discussion will mainly focus on the delivery of DNA. To introduce genetic material inside a cell, either *ex vivo* or *in vivo* transfer methods can be used. *Ex vivo* method requires the removal, genetic modification and re-administration of patient's cells. *In vivo* method however is more interesting and involves either systemic or local delivery of genetic material with for example injection. Current delivery vectors used in *ex vivo* and *in vivo* methods can be divided into two complementary categories – viral or nonviral vectors.

Viral vectors employ a genetically modified virus particle to carry the wanted DNA fragment inside them. Viruses, including for example retroviruses and adenoviruses, are naturally evolved to efficiently deliver their own DNA into cells in a pathogenic manner. Retroviruses are capable of integrating permanently within the host genome leading possibly to sustained therapeutic effect. Adenoviruses however act transiently and do not integrate in the host genome. Viral vectors that are used in gene therapy have all been manipulated to remove disease-causing genes and insert therapeutic ones, but the machinery which allows the virus to insert its genome into its host's genome is left intact.[29] Viral vectors can therefore deliver DNA with high efficiency and can possibly mediate long-term expression.[30] Neither are viral vectors troubled by one of the biggest challenges in gene therapy, the targeting of delivery to specific cells: different viruses show natural tropism to different tissues.[31] However, viruses often induce an acute immune response in their host, which has raised serious safety concerns to confront their common use. Furthermore, viral vectors have a very limited capacity considering the size of the molecule to be delivered. The risks of using viral vectors were unfortunately realised in clinical trials involving engineered adenoviruses that resulted in patient's death due to a severe immune response[32, 33] and reported findings of the risk of using engineered

retrovirus vectors that may induce incorrect insertion of the therapeutic gene within the regulatory or gene regions of a host genome leading to leukaemia.[34-37]

Nonviral methods employ their physical (carrier-free gene delivery) or chemical (synthetic vector-based gene delivery) properties to aid gene transfer or pack DNA into a form in which delivery becomes possible.[38, 39] Physical approaches such as electroporation,[40] gene gun,[41, 42] needle injection,[43] hydrodynamic delivery[44] and ultrasound,[45] utilise force to permeate cell membranes and facilitate gene transfer. Chemical methods use synthetic or naturally occurring compounds (or their combination) that bind DNA and allow the gene to cross the cell membrane (Figure 2). These vectors can increase the efficiency of delivery and are usually not plagued by immune and inflammatory response, but often exhibit low transfection efficiencies in medical applications, especially in *in vivo* delivery. Also transient expression of the transgene is a key problem that needs to be solved.

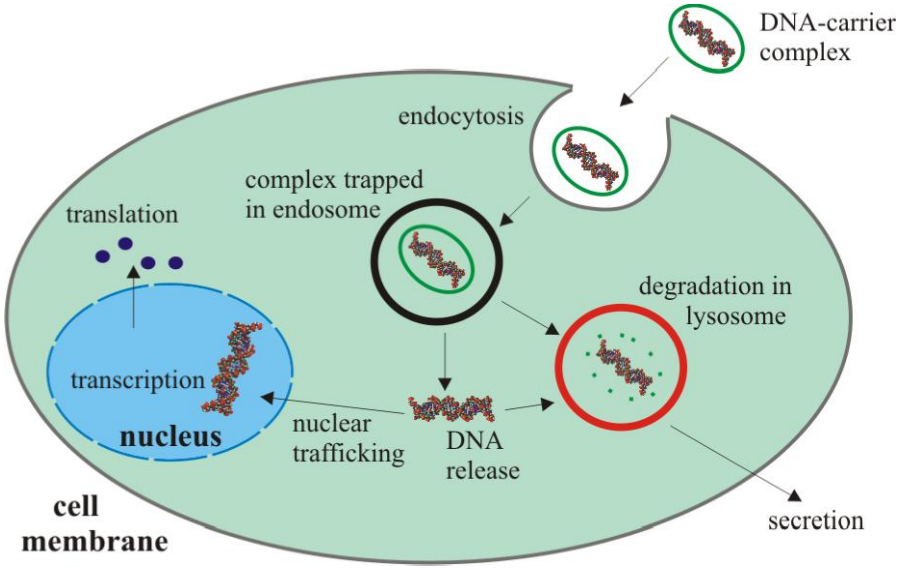

Figure 2. Simplified pathways of nonviral chemical gene delivery.[46]

Synthetic cationic systems rely on their ability to condense DNA into nanoscopic particles, which can be taken up by cells via endocytosis and delivered into the nucleus, where transgene expression can take place (Figure 2). Cationic lipids and cationic polymers are the best-studied compounds for nonviral chemical gene therapy.[46-49] Felgner and co-workers first introduced cationic lipids (N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA))[50] in 1987 and only a year later appeared a key study by Wu *et al.* who used a cationic polymer (poly-L-lysine) in gene transfer *in vivo*.[51] The much-studied polyethylenimine (PEI) was introduced a few years later in mid 90's by Boussif *et al.*[52] These landmark studies have since been flowed by numerous pioneering studies and continue to stimulate the current research.

Cationic liposomes and cationic polymers are used in slight excess so that the resulting DNA complexes have a cationic net charge that can interact electrostatically with mammalian cells, which contain surface glycosaminoglycans and proteoglycans with negatively charged chemical groups. During the endocytosis cationic lipids can spontaneously mix with the endocytic vesicles to directly increase membrane fluidity and promote release or disrupt endosome and prevent endosome maturation to lysosome,

which quickly degrades it cargo.[53] Cationic polymers however promote escape from lysosomes with a different mechanism. For example, a portion of PEI's amine nitrogen atoms can be protonated during endosome maturation when its pH drops below 6.0 and consequently the polymer can act as a proton sponge and offer buffering capacity to the lysosome. PEI can therefore protect DNA from nuclease degradation and, with the protons, bring chloride ions into the endosome, raising the osmotic pressure and cause lysosomal swelling and consequent rupture that can provide escape for the PEI-DNA complexes.[52, 54] However, there are various features in the transfection procedure that can, individually or in concert, affect the transfection efficiency. These include; the chemical structure of the transfection agent, charge ratio (the nominal number of positive charges of the polycation divided by the nominal number of negative charges present on the DNA), size and structure of the resulting lipoplexes or polyplexes, the cell line and the total amount of applied lipoplex or polyplex. These factors determine their structural morphology and net charge, which directly affect the toxicity and transfection efficiency of the procedure towards certain cells.

The importance of structural features was realised by Safinya and co-workers who have extensively studied the relationship between structural morphology of cationic lipid-DNA complexes and transfection efficiency in mammalian cells. The structural morphology of lipoplexes can be diverse and three structures have been identified: lamellar L_α^C phase,[55, 56] with alternating lipid bilayers and DNA monolayers, an inverse hexagonal H_{II}^C phase,[57] where DNA is encapsulated within inverse micellar tubules, and hexagonally arranged H_I^C phase,[58] where tubular lipid micelles are surrounded by DNA rods forming a three-dimensionally continuous substructure with honeycomb symmetry (Figure 3). However, these structures can have very different effects on transfection efficiency, for example efficiency of the inverted hexagonal H_{II}^C cationic liposome-DNA complexes is independent of the membrane charge density, but for the lamellar L_α^C complexes the data can be interpreted to a model with strong dependency between efficiency and the membrane charge density.[59]

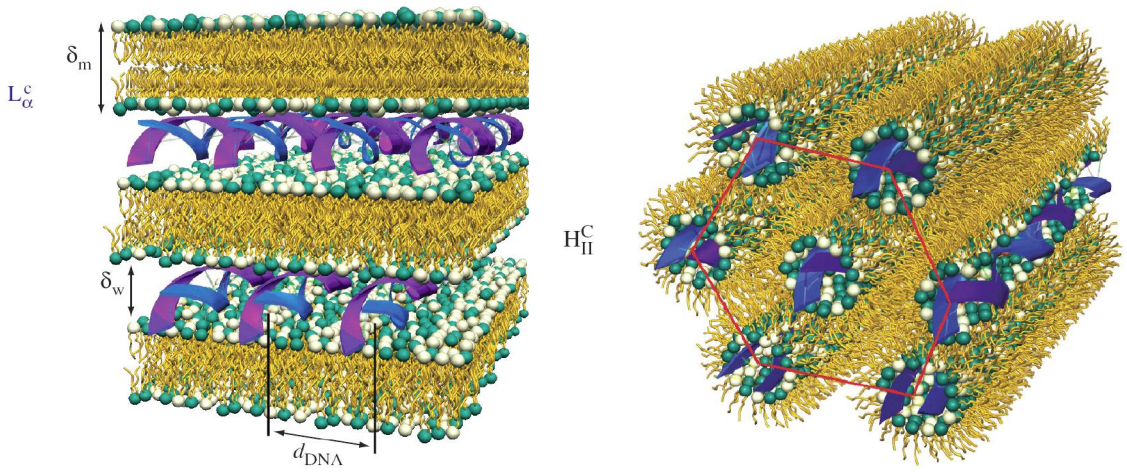

Figure 3. Self-assembled liquid crystalline equilibrium phases of cationic liposome-DNA complexes. L_α^C phase with alternating lipid bilayer and DNA monolayer and H_{II}^C phase with inverse micelles arranged on a hexagonal lattice.[55-57, 60]

DNA binding and packing is therefore one of the key features for efficient transfection with nonviral chemical methods and understanding the factors that affect the interactions between DNA and the vector is an important prerequisite for their controlled

manipulation. The ionic interaction between a protonated amine and the phosphate backbone of DNA forms the basis for most synthetic DNA binding molecules used for gene delivery. However, an individual binding unit with one pronated amine cannot efficiently bind DNA under physiological conditions and to achieve high-affinity binding larger ligands must be utilised. While the number of binding sites and size of the molecules increase, also the binding event becomes more complex to understand and tune. Therefore binding ligands that can avoid structural complexity and have a very precise structure with relatively low molecular weight are easier to study and consequently have a bigger chance to result in applications. Polyvalent interactions give rise to special phenomena such as cooperativity and multivalency and the design of such binding systems requires a detailed understanding of the delicate balance between binding entropy and enthalpy.

1.2. Multivalency

Valence of an entity has been defined as the maximum number of the same kind of separate interactions that it can form with another entity. *Multivalent* interactions can therefore occur between a host and a guest both having two or more complementary binding sites resulting in unique thermodynamic features commonly termed as *multivalency*. Conceptually related terms *cooperativity* and *chelate effect* can differ from *multivalency*, but are sometimes used in literature in inconsistent manner as a substitute for *multivalency* due to anachronistic terminology. Cooperativity can be used to describe systems which do not involve multivalency such as the influence of binding a guest at the host's binding site A on the second binding step occurring at site B of the same host. Perhaps the best known example of such cooperative system in biology is the binding of oxygen to haemoglobin subunits[61] where the binding strength of the second O_2 molecule is increased by the first one and the sum of both binding energies is higher than two times the binding energy of the first guest ($-\Delta G^{\text{poly}}_{\text{avg}} > -\Delta G^{\text{mono}}_{\text{(first)}}$). Cooperativity can therefore describe allosteric monovalent interactions that do not rely on multivalency and it can be synergistic ($-\Delta G^{\text{poly}}_{\text{avg}} > -\Delta G^{\text{mono}}$), additive ($-\Delta G^{\text{poly}}_{\text{avg}} = -\Delta G^{\text{mono}}$) or interfering ($-\Delta G^{\text{poly}}_{\text{avg}} < -\Delta G^{\text{mono}}$). Chelate effects also refers to the enhanced binding of guests to multivalent hosts, but is primarily used for small molecules (mainly metals and ions) binding to multivalent, often cyclic, hosts. Chelate effect should consequently be thought as subclass under multivalency, but not strictly related to binding of metal ions. A classic example of chelate effect is the ability of bidentate ligands (ethylene diamine, 2,2'-bipyridine) to form a more stable complex with transition metals than corresponding monodentate ligands (ammonia, pyridine).

Multivalent interactions are ubiquitous throughout biology and they play an important role in many biological recognition events.[16] To achieve effective binding, nature prefers to use multivalent interactions rather than a very strong monovalent interaction. From an evolutionary point of view this seems advantageous because it allows binding of new emerging molecules by using existing interactions rather then constructing an entirely new one and a more dynamic control of interactions. For example, if there is a rapid need for a high-affinity interaction, it is quicker to multiply the existing interactions than to develop entirely a new binding ligand. Multivalency also allows grading of biological responses or signalling by employing signal cascades where strength of the signal can vary based on the number of host-guest interactions, resulting in a range of possible signal strengths. This is

a clear advantage since a single host-guest interaction can in principle offer only an "on" and "off" type of control.

Multivalent interactions encountered in nature highlight the importance of multivalency in many biological systems. Examples of such interactions include: viral and bacterial adhesion on cell surfaces via glycoprotein recognition, cell-cell interactions between E-, P,- L-selectins and sLe^x during extravasation,[62, 63] binding of antibodies having multiple receptor sites to antigens[64, 65] and control of gene transcription by DNA binding.[66, 67] Gene expression in eukaryotes and prokaryotes is controlled primarily at the level of transcription. Most genes in eukaryotes are silent unless the multisubunit protein machinery required for transcription is specifically recruited to the TATA box at the start site of the gene. This recruitment is achieved by using transcription factors that recognise the promoter sequences, such as TATA box and additional upstream sequences, located on the 5' side of the start site. Transcription factors bind their target DNA with high affinity and sequence specificity and have an additional activation domain that aids the assembly of the RNA polymerase to the transcription complex. The DNA sequence to be recognised is often conserved and the transcription factor that recognises it can be multivalent. An example of this kind of behaviour is the gene regulation by oligomeric retinoid X receptor (RXR), which is a member of nuclear hormone receptor superfamily proteins.[68] RXR consists of two domains, a binding domain and a ligand binding domain, and functions as a transcription factor in the presence of its endogenous ligand (L), 9-*cis* retinoic acid.[69] The DNA binding domain of RXR-ligand complex (RXR-L) recognises a single stranded DNA cellular retinol-binding protein II element (CRBP-II). The striking feature of this recognition is that the ensuing transcriptive response is highly sensitive to the concentration of the transcription factor. This is because the transcription factor can function as multivalent aggregate of ligands binding to a DNA sequence with multiple binding sites. One to one binding affinity between a single RXR-L complex and a single CRBP-II element is very low, however, the binding affinity increases for an interaction between a $(RXR\text{-}L)_2$ dimer and two adjacent elements $(CRBP\text{-}II)_2$. An increase in the binding affinity is further observed when tetramers are interacting and the clearly highest affinity is between a pentamer complex $(RXR\text{-}L)_5$ and five adjacent elements $(CRBP\text{-}II)_5$ (Figure 4a). The result is that the rate of transcription is strongly activated by RXR-L multimerisation – a clear effect of multivalency.

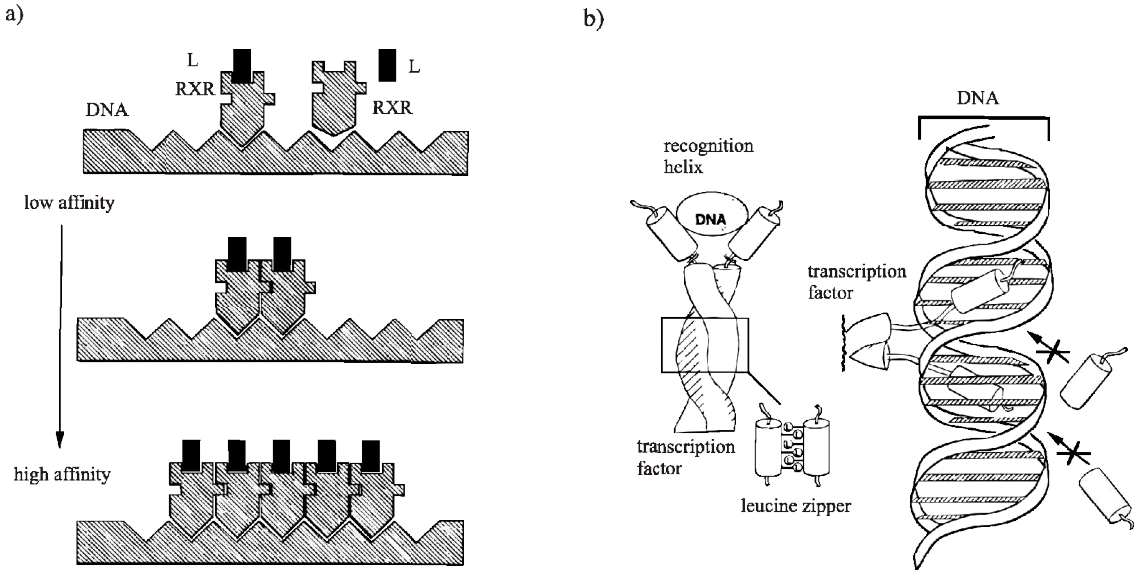

Figure 4. a) Binding of transcription factors to multiple sites on DNA: the binding affinity of RXR-L complex towards CRBP-II element is increased when the number of interacting subunits is increased.[66] Top: monomers bind DNA with low affinity, middle: dimers with higher, and bottom: pentamers with very high affinity. b) The proposed binding mode of a dimeric α-helical coiled coil leucine zipper protein to a palindromic DNA sequence.[70] Stabilising hydrophobic and van der Waals interactions between leucine residues hold the two helices together so that they can bind to the major groove of DNA.[71] Dimerisation of the two chains is required for DNA binding.

Common DNA binding motifs that occur in transcription factors include zinc finger and leucine zipper containing proteins that rely on multivalency to increase their DNA binding affinity and specificity.[70] Zinc finger relies on a β harpin-turn-α helix motif consisting of an elongated 30 residue unit, each containing two cysteines and two histidines to coordinate a zinc ion.[72] The zinc ion stabilises the structure and brings the β harpin and α helix in close proximity.[73] Leucine zipper relies on similar strategy, consisting of a stretch of close to 35 residues with a leucine at every seventh position (Figure 4b). Leucine residues help to bring two leucine zipper proteins together by stabilising the forming α-helical coiled coil. Furthermore, the leucine zippers have an approximately 30-residue long basic region at their amino-terminus, which serves as a DNA binding module.[71] Leucine zipper stabilised dimerisation is essential for achieving DNA binding. In most cases the leucine zippers do not bind DNA as monomers and dimerisation is required for the transcription factor to function.

An understanding of the structure and thermodynamics of multivalent systems is needed for the design of synthetic multivalent molecules with desired properties. Also the mechanism by which a multivalent molecule operates directly affects its potency and must therefore be accounted. Multivalent molecules can affect biological processes principally in two ways: by simultaneous binding to multiple receptor sites on biomolecules, thus serving as inhibitors, or by receptor clustering, which is a key determinant to their function as effectors.[74] Different mechanisms by which a ligand can interact with a receptor are presented in Figure 5. Monovalent ligands can commonly bind only to a single site on the rector or heterodimerise a receptor via two receptor binding faces. Mechanisms and topologies of multivalent interactions can, however, be more diverse:

- Chelate effect. Multivalent interactions in host-guest systems decrease their rate of dissociation (k_{off}), rather than increase the rate of association.
- Subsite binding. Primary binding to the receptor also promotes secondary binding interaction, which is in close proximity to the primary one.
- Steric stabilisation. Binding of a large multivalent ligand inhibits further ligands from binding by sterical blocking of the surface through the development of a large gel-like layer.
- Receptor clustering. Multivalent binding on receptors brings them to close proximity thereby altering the signalling properties of the receptors.
- Statistical effect. Multivalent ligands have a high local concentration of binding units, which promotes rebinding.

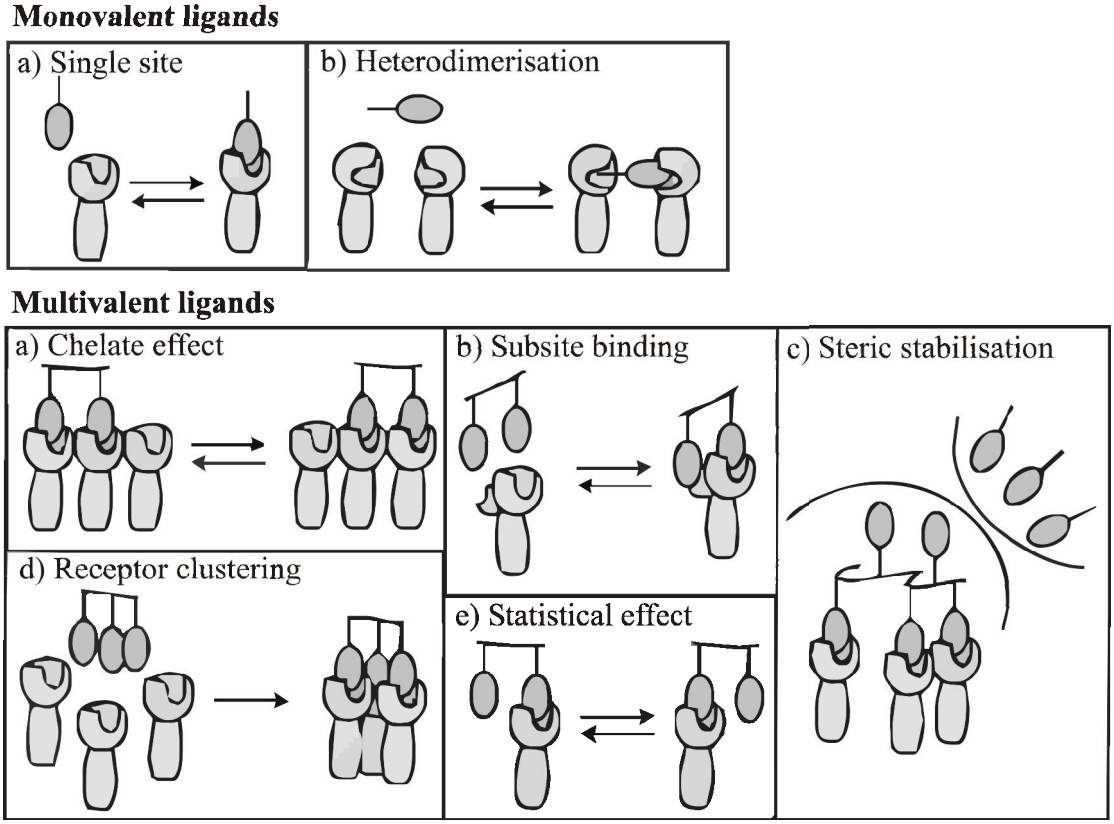

Figure 5. Binding mechanism of mono- and multivalent ligands. Monovalent ligands: a) single site binding b) receptor heterodimerisation. Multivalent ligands: a) chelate effect b) subsite binding c) steric stabilization d) receptor clustering e) statistical effects. [74]

Binding between a ligand and a receptor can be expressed by the free energy of interaction term (ΔG_N^{poly}) between N ligands and N receptors. ΔG_N^{poly} can be split into enthalpic (ΔH_N^{poly}) and entropic (ΔS_N^{poly}) components, of which the entropic term can further be presented as a sum of changes in translational ($\Delta S_{\text{trans},N}^{\text{poly}}$), rotational ($\Delta S_{\text{rot},N}^{\text{poly}}$) and conformational ($\Delta S_{\text{conf},N}^{\text{poly}}$) entropies. Also the entropy of solvation ($\Delta S_{\text{solvent},N}^{\text{poly}}$) describing the changes in the surrounding solvent molecules can be included.

$$\Delta G_N^{\text{poly}} = \Delta H_N^{\text{poly}} - T(\Delta S_{\text{trans},N}^{\text{poly}} + \Delta S_{\text{rot},N}^{\text{poly}} + \Delta S_{\text{conf},N}^{\text{poly}} + \Delta S_{\text{solvent},N}^{\text{poly}}) \quad (1)$$

The enthalpic term can either favour ($\Delta H_{\text{avg}}^{\text{poly}} < \Delta H^{\text{mono}}$) or disfavour ($\Delta H_{\text{avg}}^{\text{poly}} > \Delta H^{\text{mono}}$) binding. For example, the binding of a five subunit cholera toxin (AB_5) to its receptor, GM_1 oligosahharide moiety, is thought to be enhanced enthalpically,[75] whereas enthalpically diminished binding is encountered for example when multiple ligand receptor interactions require energetically unfavourable molecular conformations. $\Delta S_{\text{trans},N}^{\text{poly}}$ and $\Delta S_{\text{rot},N}^{\text{poly}}$ are related to molecule's freedom to move through space and rotate around itself respectively. They are both logarithmically dependent on the molecular mass of the molecule, however only translational entropy is dependent on the concentration where the entropic cost increases with decreasing concentration ($\Delta S_{\text{trans}} \propto \ln([\text{L}])^{-1}$). Conformational entropy $\Delta S_{\text{conf},N}^{\text{poly}}$ is associated with the physical arrangement of the multivalent molecule that it assumes during binding. The framework that links the multiple binding ligands together is generally flexible and does not match exactly the spacing between corresponding receptors, therefore $\Delta S_{\text{conf}} \neq 0$. Interestingly, increasing the flexibility of this linker results in increased conformational entropic cost of association, conversely the same increase of flexibility decreases ΔH_N because all ligand-receptor interactions have a smaller probability to occur with high energetic strain. Solvent interactions $\Delta S_{\text{solvent},N}^{\text{poly}}$ are particularly important for ions in water, as they provide the major driving force for ion solvation. Also the release of water molecules that bind strongly together contribute to the occurring interactions.

Investigating the thermodynamics of a multivalent system can be very challenging. Kitov *et al.* have presented that the analysis of the thermodynamic parameters of multivalent interactions with a multimeric receptor requires a special thermodynamic model, which consisting of three elements: free binding energy of the initial single ligand-receptor interaction, free binding energy of the other ligands in the same molecule on the receptor and probability of association and dissociation of individual ligand branches.[12]

$$\Delta G_{avidity}^{o} = \Delta G_{\text{inter}}^{o} + \Delta G_{\text{intra}}^{o} \sum_{i=1}^{i_{\max}} w_i (i-1) + RT \sum_{i=1}^{i_{\max}} w_i \ln(w_i / \Omega_i) \qquad (2)$$

$\Delta G_{\text{inter}}^{o}$ and $\Delta G_{\text{intra}}^{o}$ are the two microscopic binding energies corresponding to the inter- and intramolecular interactions. The first term $\Delta G_{\text{inter}}^{o}$ corresponds to the free energy of the first monovalent interaction $\Delta G_{\text{mono}}^{o}$ and is separated from the second term $\Delta G_{\text{intra}}^{o}$, which subsequently describes the maximal number of additional intramolecular interactions ($i_{\max}$ –1), therefore $\Delta G_i^{o} = \Delta G_{\text{inter}}^{o} + (i-1)\Delta G_{\text{intra}}^{o} - RT \ln \Omega_i$ applies, where ΔG_i^{o} represents the free energy level of a degenerate state in ligand-receptor complex with i number of interactions. A partial average over all bound states of the receptor gives the weight coefficient probability w_i of an individual i^{th} bound level. The statistical term $-R\sum w_i \ln(w_i / \Omega_i)$ in equation (2) can be expressed in entropy units and has been regarded as avidity entropy ($\Delta S_{\text{avidity}}^{o}$), which is a measure of disorder in the distribution of microscopically distinct complexes. The degeneracy of the bound states (Ω_i) is dependent on the topology of the interactions and reflects an ensemble of microscopically distinguishable ligand receptor complexes, rather than an individual ligand-receptor molecule. G. Ercolani has drawn similar conclusions in his assessment of cooperativity in self-assembly, where he also suggests that inter- and intramolecular processes should be

considered as two distinct groups, although only virtually identical processes described by equilibrium constants having the same dimensions should be compared.[76] These type of models can be used for the analysis of multivalent interactions and the prediction of inhibition levels of multivalent receptor by a multivalent ligand as well as rational design of multivalent compounds for desired purposes.[74] Although elaborate efforts have been made to understand multivalency, the strong increase of binding affinity is still not fully understood in terms of enthalpy and entropy.

How to choose the best scaffold for multiple binding ligands? Different classes of scaffolds include covalent frameworks such as low molecular weight compounds, dendrimers and dendrons, globular proteins, linear polymers and polydisperse polymers. Non-covalent frameworks include for example liposomes. Kiessling and co- workers have studied the influence of multivalent ligand architecture (low molecular weight compounds, polyamidoamine (PAMAM) dendrimers, bovine serum albumin protein, linear polymers or polydisperse polymers) on the receptor-ligand binding mechanism.[77] In this key study, four different assays were used to assess the inhibitor and effector function of different classes of multivalent compounds. Solid-phase binding assay was used to study binding inhibition and quantitative precipitation, turbidity measurements and quenching of fluorescence emission were used to investigate effector function. In general, low molucalr weight compounds and globular proteins were observed to be poor inhibitors for binding and poor effectors due to insufficient receptor clustering. Linear polymers and polydisperse polymers were, however, much more effective inhibitors and effectors. PAMAM dendrimers fall in between of these two groups in terms of their ability to cluster receptors. Dendrimers and dendrons however posses other inherent advantages, such as relatively low molecular weight, precisely defined structure, multivalent surface and the possibility for easy structural variations thereby making them interesting scaffolds for biological applications.

1.3. Dendrimers and Dendrons in Gene Therapy

Dendrimers are highly branched polymers exhibiting a symmetric monodisperse treelike structure. A dendron, however, is an asymmetric half of a dendrimer. Both dendrimers and dendrons consist of three main structural components (Figure 6):

- Central core (**C**), the inner centre of the molecule. Also referred to as the focal point.
- Branched units (**G_1**, **G_2**), which define the size, rigidity and density of the dendrimer.
- Surface groups (**S**), offering numerous possibilities for functionalisation.

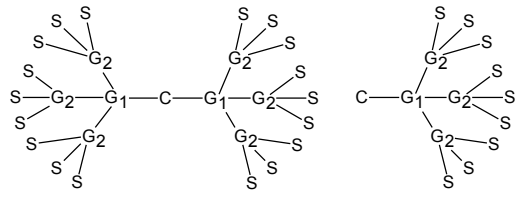

Figure 6. Structure of dendrimers and dendrons.

Branched units are attached to the core in an iterative manner, to form layers called “generations”. Each successive repeat unit along all branches forms the next generation of growth: G1, G2, G3 and so on. There are two common approaches to dendrimer synthesis, divergent and convergent. In the divergent approach branching monomers are introduced one generation at a time, beginning from the core and ending at the periphery.[78] However, convergent synthesis begins from the outer surface shell of the target molecule and ends, after an iterative synthetic procedure, at the central core.[79] Both synthetic strategies possess relative advantages and disadvantages and the method of choice always depends

on the structure of the target molecules, the synthetic methods available for growth and the specific synthons used in the construction of the dendritic framework.

The branched backbone can subsequently be functionalised with appropriate surface groups, and indeed a very high density of functional groups is achievable due to the branching structure. Therefore, the surface can be designed to have definite physical and chemical properties. The exactly defined branched superstructure offers specific advantages, for example a globular shape, radially controlled chemical structure, variable inner volume and multivalent surface. Asymmetric dendrons have an additional advantage; the central core can be substituted with desired functionality.

Dendrimers can also be prepared by a variety of self-assembly processes, such as, supramolecular coordination chemistry or hydrogen bonding.[15, 80] Coordination chemistry is synthetically easy to access, and with appropriate choice of metals, can afford durable structures. Dendritic structures can be prepared for example by using 2,3-bis(2-pyridyl)pyrazines as bridging ligands, bipyridines as terminal ligands and Ru(II) or Os(II) as coordinating metals (Figure 7a).[81] These complexes exhibit extraordinarily large molar absorption coefficients in the UV and visible spectral region. Furthermore, they contain also a great number of redox-active centres, making these complexes applicable to multielectron-transfer catalysts and photochemical molecular devices.

Hydrogen bond mediated self-assembly can also be utilised to form mesomolecular dendritic assemblies with high stabilities and helical arrangement due to the bonding strength and high degree of directionality. For example Zimmerman *et al*. prepared a family of Fréchet-type dendrimers capable of assembly through the rigid tetracarboxylic unit at the focal point (Figure 7b).[82] More strikingly, the information programmed into dendritic branches at molecular level controls directly the morphology of the formed assembly – a dendritic effect. Higher dendritic generation was found to increase the stability of cyclic hexamer over linear aggregation (supramolecular polymer).

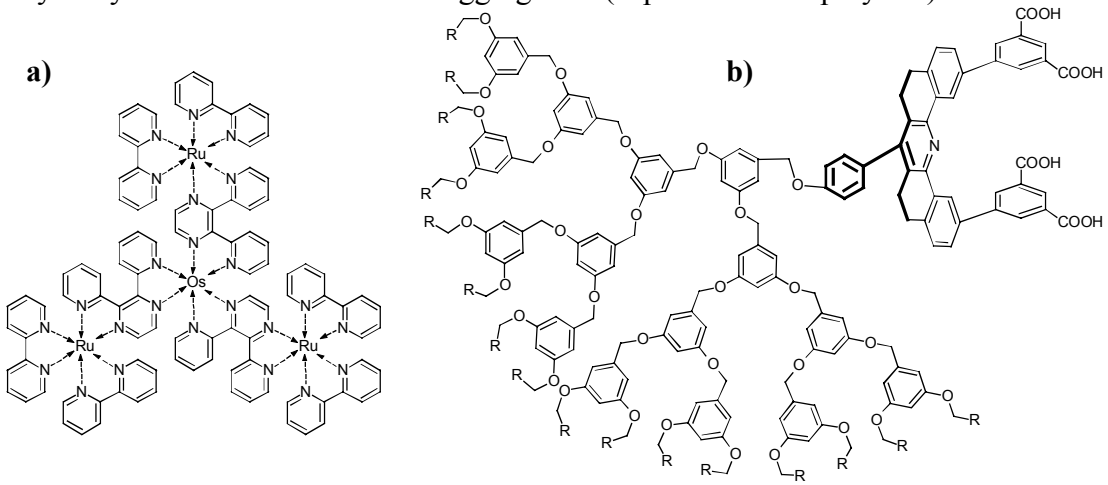

Figure 7. a) Self-assembled heterometallic dendrimer.[81] b) Isophtalic acid functionalised dendron.[82]

A second way to utilise self-assembly is to use dendritic building blocks and their dendron-dendron interactions to generate extended arrays through hierarchical self-assembly. This kind of process usually leads to nanoscale structures in gel phase, such as fibers and sheets, which also express their properties on a macroscopic scale, leading to interesting materials properties.[83] For example, fibrillar assemblies are recurrently found

structures in nature, appearing with many kinds of functionalities and as part of complex structures. Their research is of intense current interest because of their relevance in neurodegenerative diseases, such as Alzheimer's and Creutzfeldt-Jakob diseases.[23]

Supramolecular fibers assembled from Fréchet-type dendrons with a dipeptide (Tyr-Ala) focal point have been reported by Aida and co-workers. These structures rely on well-organised complementary supramolecular interactions which give rise to a directional assembly process (Figure 8).[84] Higher generation dendritic branching was required for effective gelation at low dendron concentration (1.0 mM). Detailed structural analysis revealed fibrous nanostructure for some gelators while others showed 30-60 nm wide nanoribbons (sheet structure). Interestingly, some of the gels indicated a helically twisted hydrogen bonded arrays (fibers) in circular dichroism analysis.[85]

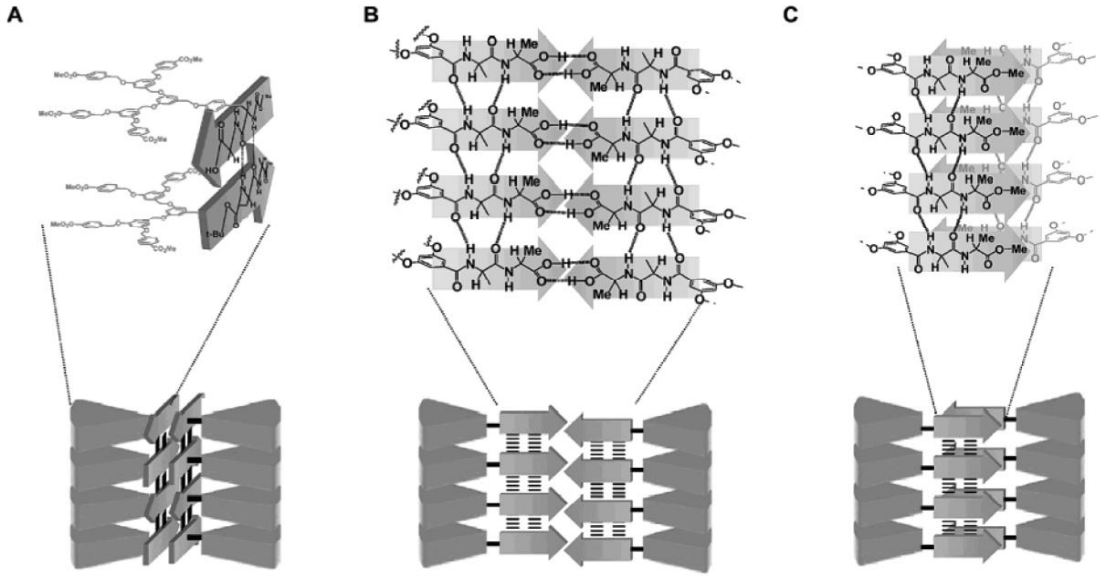

Figure 8. Proposed self-assembled structures of the dipeptide-core dendritic macromolecules.[85]

There has been enormous interest in using dendritic molecules or materials based upon dendritic building blocks for biological applications.[16, 86-94] Dendrimers and dendrons have widely been used as multivalent scaffolds to organize manifold binding ligands for the recognition of wide range different compounds, for example the multivalency principle in the binding of saccharides to proteins on cell surfaces is now well established.[11, 74, 95, 96] In order to utilise dendrimers for energetically favourable binding interactions, they should be designed in such a way where the number of binding interactions is maximised while internal strain in the bound molecules is minimised. Both enthalpic and entropic factors must be carefully considered because, enhanced binding energy of multisite attachment must overcome the steric strain induced by the binding event. The high local concentration of binding units and easy structural tunability of dendrimers can provide means to investigate how different aspects of, for example, receptor clustering can be independently influenced by multivalent ligand architecture. Dendrimers have been observed to rapidly induce receptor clustering, although the orientation of the receptors was such that would allow their function as effectors. This is probably due to the unfavourable distance and orientation between receptors in the complex.[77] These results demonstrate that not only the binding ligands but also the multivalent ligand architecture contribute to the binding modes and affinities. Although the principles underlying the multivalency principle can be understood to some extent, a rational design of binding scaffold can be notoriously difficult and efficient binding ligands are often discovered by sheer trial and error.

Dendrimers are often used as DNA binding agents and consequently their ability to transfect DNA has been widely studied.[97, 98] DNA binding and packing is one of the key features for efficient transfection. The interaction between a single protonated amine and the phosphate backbone of DNA forms the basis for most DNA binding molecules. Spherical polyamidoamine (PAMAM) dendrimers are relatively straightforward to synthesise and have a positively charged polyamine surface and as a consequence they have been studied extensively.[99, 100] Increasing molecular weight of PAMAM dendrimers has been found to amplify the transfection efficiencies. Indeed, a molecular weight greater than 116 000 Da was determined to be optimal. Heat fracturing of dendrimer framework results in a higher transfection efficiency, potentially as consequence of their greater flexibility and ability to compact DNA.[101] Attachment of polyethylene glycol (PEG) units has been reported to enhance stability, transfection efficiency and circular half-life of PAMAM dendrons.[102, 103] Pegylated PAMAM dendrons also exhibited very low cytotoxicities, although the overall transfection efficiency still remained low. In search for better transfection agents also poly(propylene imine) [104] (PPI) and dendritic L-lysine[105] have been studied. Park and co-workers took an interesting approach and combined a linear polyethylene glycol and dendritic poly(L-lysine) to form a architectural co-polymer.[106] This novel block co-polymer could self-assemble with plasmid DNA at physiological conditions, forming a compact and water-soluble polyionic complex. The formed complex was studied by atomic force microscope (AFM) and found to take a globular shape with a relatively narrow size distribution (see Figure 9). Nuclease resistance and gel electrophoresis were used to confirm the binding and packing results.

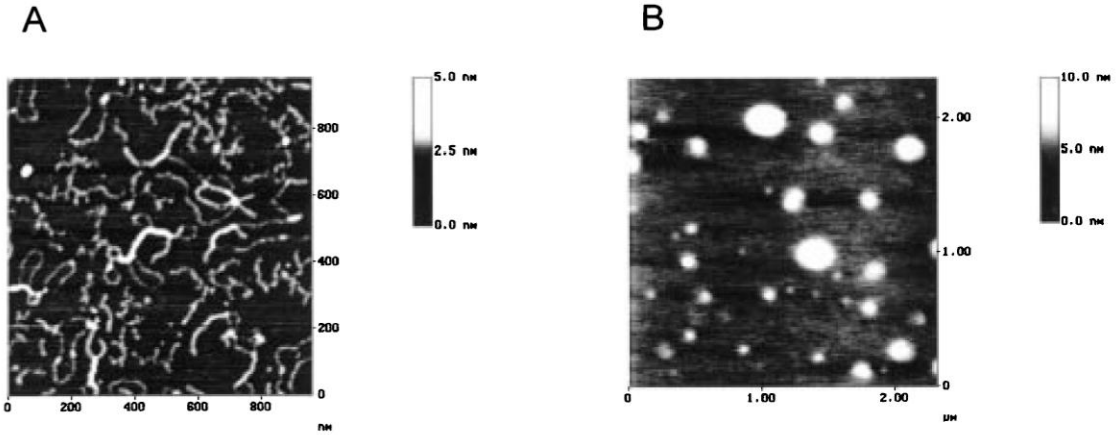

Figure 9. a) Plasmid DNA and b) globular complexes formed between DNA and dendritic poly(L-lysine) / PEG block co-polymer as studied by AFM.[106]

With the aim of producing more programmable supramolecular architectures of defined and controllable composition in space, Diederich and co-workers have reported rationally designed amphiphilic Newkome-type dendrimers for gene delivery.[107] Relying on the low toxicity, geometric tunability, and ease of multiple functionalisation of cationic dendrimers on the one hand and the classic self-assembly of amphiphilic molecules on the other, they developed a set of molecular building blocks to prepare amphiphilic dendrimers. The rational design led to structures with rigid cores, high cationic charge density on one side of the dendrimer surface and alkyl tails on the other (Figure 10). Indeed, these amphiphilic dendrimers were found to have very high transfection efficiencies, which were directly modulated by the size and number of alkyl tails and cationic groups in the dendrimer. Transfection efficiency of these compounds even exceeded that of Superfect^{TM}, which is an efficient commercial transfection agent.

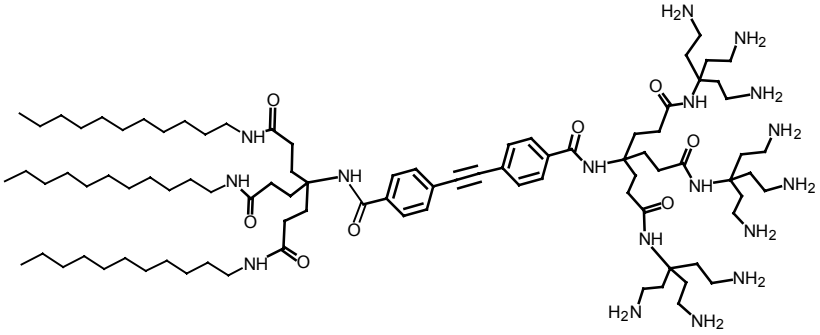

Figure 10. A tailored self-assembling amphiphilic dendrimer for efficient gene transfection.[107]

All of the above mentioned examples rely on the DNA binding function of the dendritic vector. However, since a simple DNA binding molecule will most likely fail in delivering DNA to a specific site at specific time *in vivo*, also other 'smart' functionalities have been incorporated in nonviral vectors in order to enhance transfection efficiency and targeting. Gene delivery vectors with smart properties could allow real-time control of delivery or the gene transfection in the body. These types of vectors are currently under intense research and could open new important ways to develop the field. Strategies for making smart dendritic vectors with desired functionalities include principally two approaches: targeting vectors by using cell-surface receptors and release of DNA using light or other external stimulus. However, smart DNA delivery vectors based on dendritic molecules remain largely unexplored.

Dendrimers that target DNA via receptor mediated endocytosis commonly consist of two covalently liked segments:[108] a segment that is a ligand for a cell surface receptor and a cationic DNA binding segment (Figure 11). Upon mixing with DNA the ligands are hypothesised to bind DNA and the targeting units remain exposed on the surface. The ligand can be chosen to promote internalization via receptor mediated endocytosis to a cell type that has the appropriate cell surface receptors to recognise the ligand.

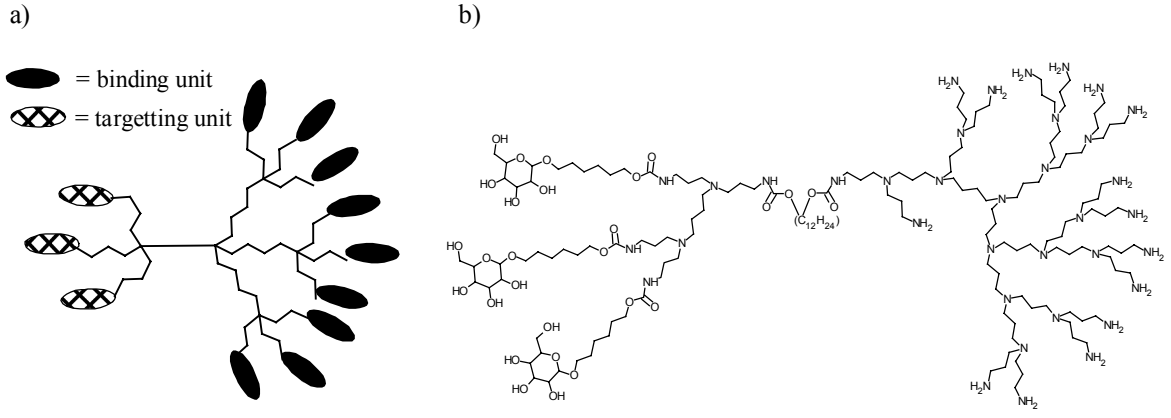

Figure 11. a) Structural design of a bifunctional multivalent dendron containing both binding and targeting ligands. b) Bifunctional dendron with 15 surface amines and three galactosyl residues used for efficient targeting of gene delivery to liver hepatocytes.[109, 110]

Kim *et al.* have studied a series of bifunctional dendrimers consisting of a DNA binding amine wedge and galactosyl functionalised wedge.[109, 110] They concluded that a

dendrimer with 15 surface amines and three galactosyl residues was capable of delivering DNA specifically to liver via asialoglycoprotein receptors on hepatocytes, being also the optimal vector of the compounds studied. These types of dendritic vectors can be designed in rational way to promote efficient DNA binding and target-specific gene delivery *in vivo*.

Dendritic systems that can bind DNA and respond to external stimuli to control or release DNA include azobenzene and phthalocyanine dendrimers. It was found that the azobenzene dendrimer could respond to UV light and change its zeta potential and size. It was further demonstrated that the DNA binding ability of the dendron was dependent on its surface charge – dendrimer with higher surface charge also had higher affinity towards DNA.[111] This is an important result since it shows that DNA binding affinity of a dendrimer can directly be controlled by external stimulus. The first successful photochemical-internalization-mediated gene delivery *in vivo* was published by Kataoka and co-workers.[112-114] They have developed a phototriggered system for in vivo DNA delivery, in which the vector is composed of three components: a photosensitive anionic phthalocyanine dendrimer (Figure 12a), which provides photosensitizing action, and DNA packaged with cationic peptides which drives the third DNA payload towards the nucleus of a cell after it has been released. Peptide-DNA polyplex can be released from the ternary complex by laser irradiation at the visible wavelength, because laser irradiation on the phthalocyanine dendrimer can induce photodamage to the endosomal membrane and thus enhance endosomal escape (Figure 12b). Indeed, the *in vitro* transgene expression was enhanced more than 100-fold by photochemical treatment and *in vivo* subconjuctival injection of the ternary complex in an animal model showed transgene expression only in the laser-irradiated site. This system presents the state-of-the-art in photochemical enhancement of transgene expression by dendritic compounds.

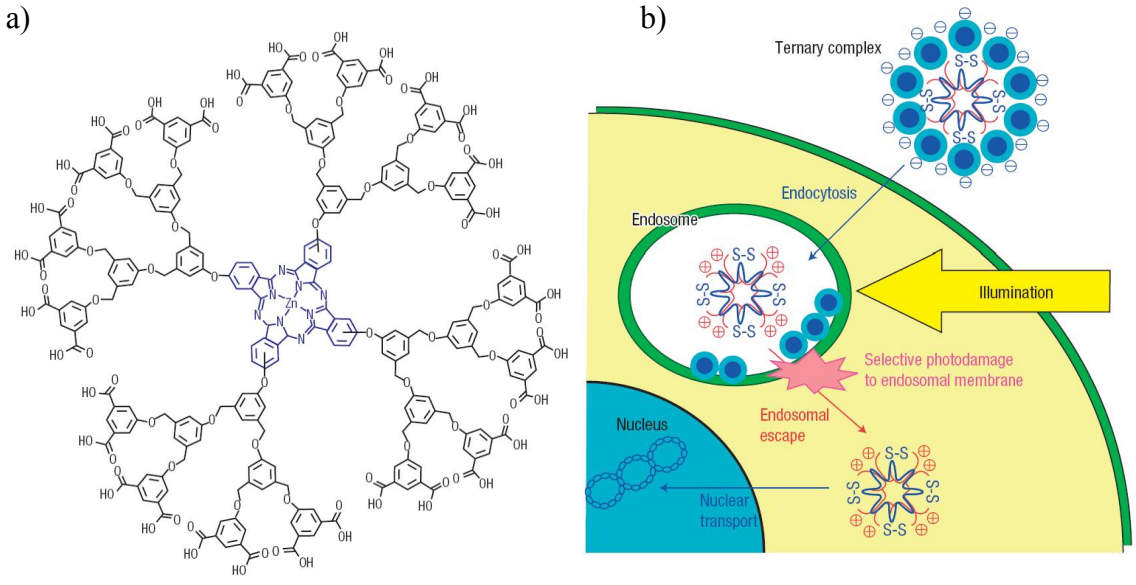

Figure 12. a) Structure of the anionic phthalocyanine dendrimer. b) A schematic presentation of the photochemical internalisation mediated gene delivery. Ternary comples is designed to enter the cell by endocytosis. Phthalocyanine dendrimer can induce selective photochemical damage to endosomal membrane.

Other recent studies, although not based on dendritic molecules, have looked to the development of functional DNA binding systems, which include quantum-dots,[115] photosensitators[116], nanoparticles[117] or bioconjugates.[118, 119] To achieve sufficient transfection efficiencies and cell-specific targeting, protein–polymer conjugates containing a cationic polymer such as polyethyleneimine (PEI) or poly(L-lysine) and an immunoglobulin have been developed.[120, 121] These conjugates rely on the ability of cationic polymers to bind and to compact DNA, with the antibody being selected to facilitate receptor-mediated gene delivery into various cell types.[122] Thus, it is possible to design very efficient conjugate vectors that possess distinct mechanisms to accomplish DNA binding and cellular targeting.

1.4. An Outline of the Thesis

The results presented in this thesis demonstrate that low-molecular-weight dendrons are capable of high-affinity DNA binding in a generation dependent manner under physiological salt conditions (article I) and are capable of modest gene delivery (article II). Spatial and temporal control over the DNA binding was achieved by synthesising dendrons in which the surface groups are attached via photolabile *o*-nitrobenzyl linker (article III). Furthermore, it has been shown that these dendrons can be attached on larger biomolecules and that their DNA binding ability is fully and directly transferred to other nanoscale objects e.g. proteins (article IV and V). All these different approaches are presented schematically in Figure 13.

Article I describes progress in optimising DNA binding and developing low molecular mass dendrons with very high affinities for DNA – such systems would be particularly useful for DNA encapsulation and protection. We studied Newkome-type polyamine dendrons, which have multivalent spermine arrays on their surfaces to increase the binding efficiency. Such monodisperse systems enable an understanding of structure-activity relationships, and additionally, have a greater chance of being licensed for therapeutic applications in the longer term. In article II we studied the gene transfection properties of these dendrons and found out that the dendrons transfect DNA only in the presence of chloroquine, which promotes endosomal escape. The DNA binding affinity of these dendrons might actually be too strong for efficient transfection and we therefore developed systems where DNA can be released by external stimulus. In article III we describe how light can be used to release DNA from dendrons by degrading and charge switching multivalency. DNA binding compounds that can be manipulated by light are especially interesting in eye targeted non-viral gene therapy. Article IV and V describe *N*-maleimido cored dendrons that can be attached onto protein surfaces in site-specific manner to yield exactly defined one-to-one protein-polymer conjugates, where the number of dendrons and their attachment site on the protein surface is precisely known. As suspected, the resulting protein-dendron conjugates bind DNA with high affinity. Further studies in gene transfection, cytotoxicity and self-assembly establish relevance in gene therapy as well as surface adhesion and patterning.

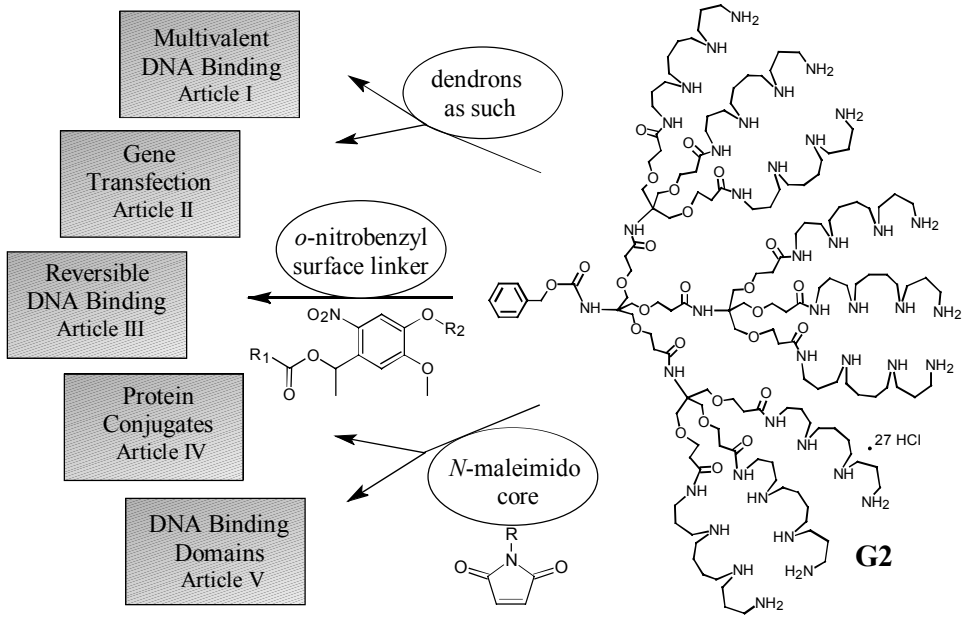

Figure 13. Schematics of the different dendritic derivatives used in this Thesis to bind DNA.

2. HIGH-AFFINITY MULTIVALENT DNA BINDING BY USING POLYAMINE DENDRONS

DNA constitutes a particularly interesting target for nanotechnological exploitation. High affinity binding of DNA is useful for protecting DNA and ultimately delivering genetic information into cells. Non-covalent interactions between dendritic macromolecules and DNA are therefore of considerable current interest. In general, higher generation, or structurally fractured, systems are usually more effective for DNA binding and delivery. However, it would be advantageous to device low-molecular-weight compounds capable of high-affinity DNA binding. Most dendrimers and dendrons are utilised to bind DNA by using protonated amine surface groups that form ionic interactions with the anionic phosphate backbone of DNA. The interaction between a single protonated amine and a phosphate is relatively weak, and must compete with salt binding under biological conditions. Biology therefore uses tetraamines, such as spermine, to enhance and achieve DNA binding. Synthetic spermine derivatives are also widely used for applications in DNA binding and delivery. However, although spermine is better than an isolated amine for binding DNA, the interaction is still relatively weak, and consequently, spermine struggles to compete with DNA-bound inorganic cations and loses its DNA binding ability for example at higher salt concentrations.

In the following chapter it is demonstrated that the whole is more than the simple sum of its parts. The synergistic multivalency effect of having multiple amine groups organised onto single subunit can induce a binding affinity that is three orders of magnitude larger when compared to individual binding units. However, even though the dendrons bind DNA with extremely high affinity, the *in-vitro* gene transfection studies show only weak transfection efficiency.

2.1. Low-Molecular-Weight Dendrons for DNA Binding (Article I)

This study presents multivalent dendritic spermine constructs with extremely high, salt independent binding affinities for DNA. Synthesis of the target molecules was achieved using divergent route, which was based on an efficient method to form orthogonally protected second-generation dendrons, recently introduced by Cardona and Gawley.[123] The dendritic moiety for all target molecules was chosen to be a biologically compatible trifurcated Newkome-type ether dendrimer, based upon tris(hydroxymethyl)amine. This framework is easy to synthesise and it allows minimisation of steric hindrance in the second-generation dendrimer. The branches are also very flexible and therefore should encourage complexation with DNA. This very same structure forms the basis of all the dendrons presented in this Thesis. Focal point of the dendron was protected with a benzyloxycarbonyl (Cbz) group, which is easy to remove enabling the attachment of different functional groups. The synthetic flexibility provided by this latent reactive site at the focal point is an inherent advantage of the dendron and provides vast potential for the development of different DNA binding systems.

It was first necessary, however, to selectively protect the spermine to enable its clean coupling to the periphery of the growing dendron. One of the primary amines of spermine was regioselectively protected using the methodology of Blagbrough and Geall to give the protected amine, which was subsequently reacted with an excess of *t*-butoxycarbonyl (BOC) anhydride to protect the remaining amines.[124] Treatment with conc. aq. ammonia

yielded the asymmetrically BOC-protected spermine **1** (see the attachment at the end of the thesis for graphical illustration, bold numbers refer to this scheme).

First and second generation Cbz–protected dendrimers **G1**, **G2** and **G0** model compound (Figure 14) were efficiently and conveniently synthesised using carbodiimide-hydroxybenzotriazole (DCC/HOBt) coupling chemistry and orthogonal protections. Synthesis of the dendritic backbone began with 1,4-Michael addition of tris(hydroxymethyl)aminomethane (Tris) to *tert*-butyl acrylate, producing the core structure with the free amine **4**. The primary amine was then reacted in good yield with benzylchloroformate to afford Cbz-protected focal point (compound **5**). Hydrolysis of *t*-butyl protected acid groups of compound **4** with formic acid gave the tri-acid **6**. Tri-acid **6** and protected spermine **1** were then coupled using standard DCC and HOBt peptide coupling under basic conditions to provide fully protected first generation dendrimer **7**. The crude mixture was first purified with silica column in order to eliminate the excess of DCC and HOBt. The residue was then concentrated and further purified with preparative GPC to remove the side product dendron with only one or two branches. Deprotection of the spermine BOC groups with HCl gas in methanol afforded target compound **G1** in quantitative yield. **G0** model compound and its precursor **3** were constructed from polyether **2** and protected spermine **1** with similar DCC/HOBt coupling and HCl treatment. The approach followed for the synthesis of second generation target compound **G2** was based on the peptide coupling of **4** and **6** with DCC and HOBt. Deprotection of the dendron periphery, the following functionalisation with spermine and its deprotection were carried out as described for **G1**.

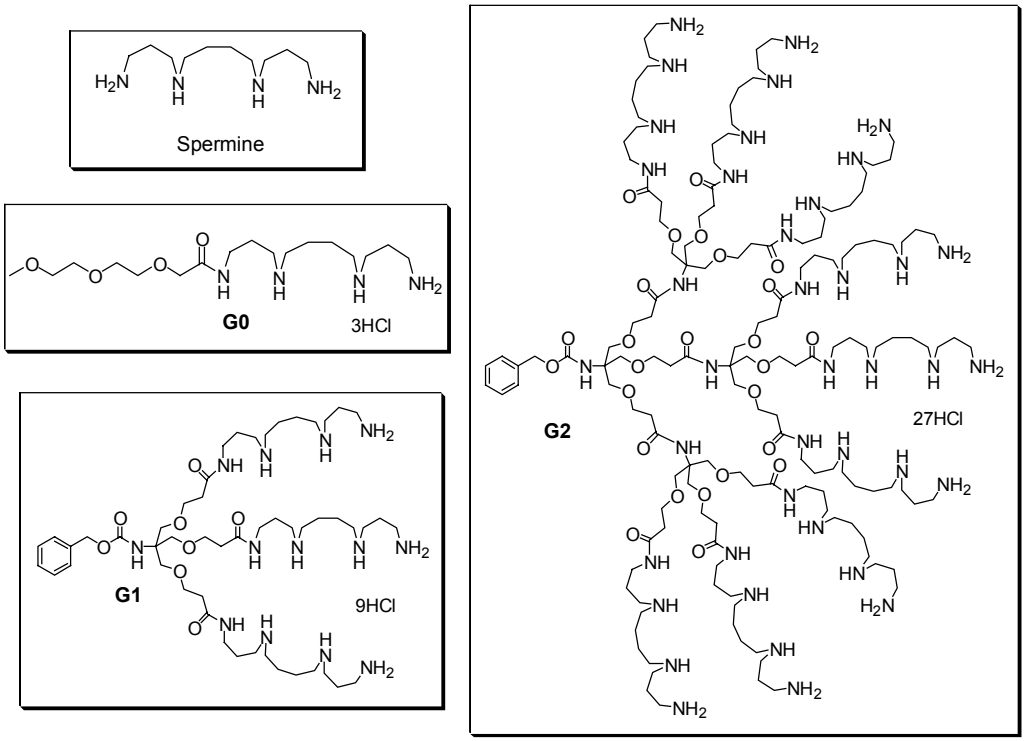

Figure 14. Spermine and target spermine derivatives **G0**, **G1** and **G2**.

Ethidium bromide displacement assay was utilised to study the binding of the spermine derivatives to DNA. This assay measures the competition between the ligands and EthBr for binding to DNA – as EthBr is displaced by the ligands, its fluorescence, which is enhanced when bound to DNA, decreases in intensity. The data obtained are presented in

terms of C_{50} and CE_{50} values (Table 1). C_{50} values report the concentration of polyamine causing a 50% decrease in fluorescence intensity. CE_{50} values represent the 'charge excess'. Charge excess is defined as the nominal "number of positive charges" of the polyamine divided by the "number of negative charges" present on the DNA.

At 9.4 mM NaCl concentration spermine binds to DNA with moderate strength (C_{50} = 1.33 μM, CE_{50} = 5.3), but as the NaCl concentrations is increased to a physiological 150 mM level, spermine virtually loses its DNA binding ability (C_{50} = 390 μM, CE_{50} = 1560). Compound **G0** showed similar, if slightly weaker, DNA binding. This was expected, as one of the primary amines of spermine has been converted into an amide, which is incapable of protonation, and **G0** should therefore exhibit weaker electrostatic interaction with polyanionic DNA.

Larger dendrons **G1** and **G2** with three and nine spermine units respectively showed significantly enhanced DNA binding. **G1** could efficiently bind DNA under low salt conditions (C_{50} = 76 nM, CE_{50} = 0.68). Notably, the affinity for DNA is considerably more than three times higher than that of **G0**. This indicates that the organisation of three spermine units on the dendritic framework enables DNA binding activity that is more than the simple sum of its individual parts – the multivalency principle[3] in operation. When the NaCl salt concentration is increased to 150 mM the binding affinity of **G1** is somewhat affected but still shows reasonable binding under these conditions (C_{50} = 300 nM, CE_{50} = 2.70).

G2 has similar DNA binding affinity as **G1** at low salt concentration (C_{50} = 30 nM, CE_{50} = 0.81), however the binding affinity of **G2** is not altered by the increase in salt concentration and the binding remains just as strong (C_{50} = 28 nM, CE_{50} = 0.76). The binding is therefore salt independent – a pro-active dendritic effect. The multivalent system can therefore compete with Na^+ cations for binding sites on the surface of the DNA helix. Indeed, this proves that the strategy of organising spermine units into a well-defined multivalent array has considerable power (Figure 15).

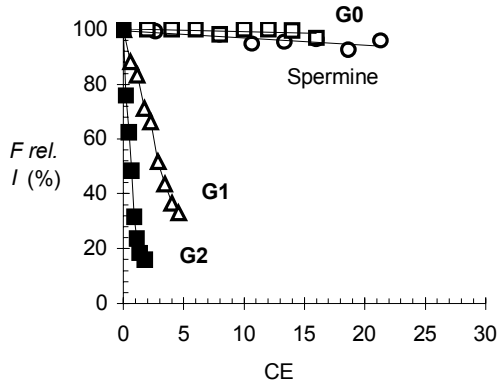

Figure 15. Fluorescence titration profiles for the addition of spermine, **G0**, **G1** or **G2** to a DNA solution in buffered water (pH 7.2) in the presence of 150 mM NaCl.

Table 1. Results for spermine, **G0**, **G1**, **G2**, **HFBI**, **HFBI-G1** and **HFBI-G2** from an ethidium bromide displacement assay.[a]

Compound	Molecular weight	Nominal charge	CE_{50}/9.4 mM NaCl	CE_{50}/150 mM NaCl
spermine	202.3	4+	5.3	>400
G0	363.3	3+	60	>400
G1	1024.4	9+	0.7	2.7
G2	3088.3	27+	0.8	0.8

[a] Total added polyamine solution did not exceed 5% of the total volume; therefore corrections were not made for sample dilution. Results are an average of three titrations.

DNA binding affinities were verified by gel retardation assay. Both spermine and the **G0** model compound failed to bind DNA and retard its migration, even at mass ratios of 1:100 (DNA:polyamine) (Figure 16). In contrast, dendritic spermine derivatives **G1** and **G2** both effectively retarded the migration of DNA at mass ratios of 1:1 (DNA:polyamine) and above. This highlights the cooperativity of the DNA binding afforded by using a dendritic scaffold for the placement of multiple spermine groups, and also confirms the results from the ethidium bromide displacement assay.

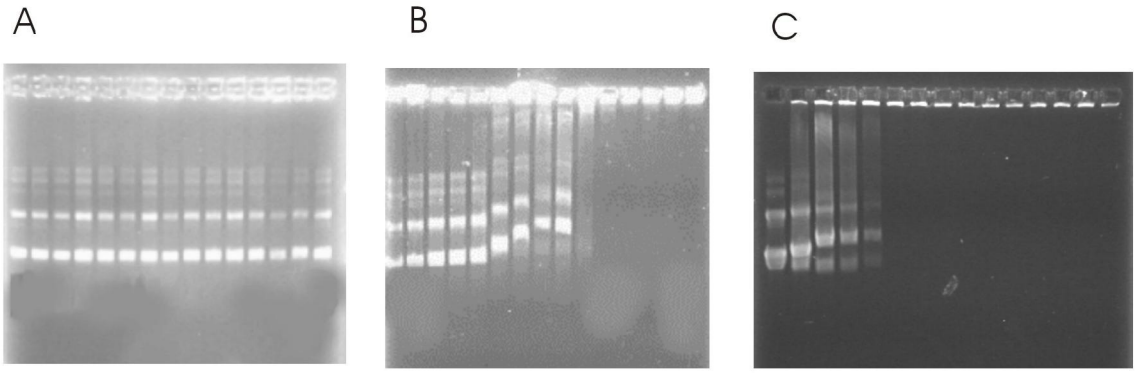

Figure 16. Agarose gel electrophoresis of polyamine/DNA complexes. (A) **G0** (polyamine:DNA, w:w): lane 1, 0:1; lane 2, 6:1; lane 3, 7:1; lane 4, 8:1; lane 5, 9:1; lane 6, 10:1; lane 7, 20:1; lane 8, 30:1; lane 9, 40:1; lane 10, 50:1; lane 11, 60:1; lane 12, 70:1; lane 13, 80:1; lane 14, 90:1; lane 15, 100:1. (B and C) **G1** and **G2** respectively (polyamine:DNA, w:w): lane 1, 0:1; lane 2, 0.1:1; lane 3, 0.2:1; lane 4, 0.3:1; lane 5, 0.4:1; lane 6, 0.5:1; lane 7, 0.6:1; lane 8, 0.7:1; lane 9, 0.8:1; lane 10, 0.9:1; lane 11, 1:1; lane 12, 2:1; lane 13, 3:1; lane 14, 4:1; lane 15, 5:1.

Transmission electron microscopy (TEM) was used to visualise the assembly of DNA-dendrimer complexes (Figure 17). With spermine-DNA complexes (CE=1.8) large unsymmetrical aggregates ca. 250 nm in diameter were observed. Compound **G0**, however, led to little or no compaction of DNA under the same conditions. On the other hand, **G1** and **G2** (CE 2.7) both condensed plasmid DNA into well-defined approximately spherical nanoscale complexes (**G1** ca. 100 nm, **G2** ca. 400 nm) with no free plasmid being detected. The size range of the aggregates formed was relatively large. Nonetheless, these observations indicate that compounds **G1** and **G2** efficiently bind DNA and condense it into spherical complexes.

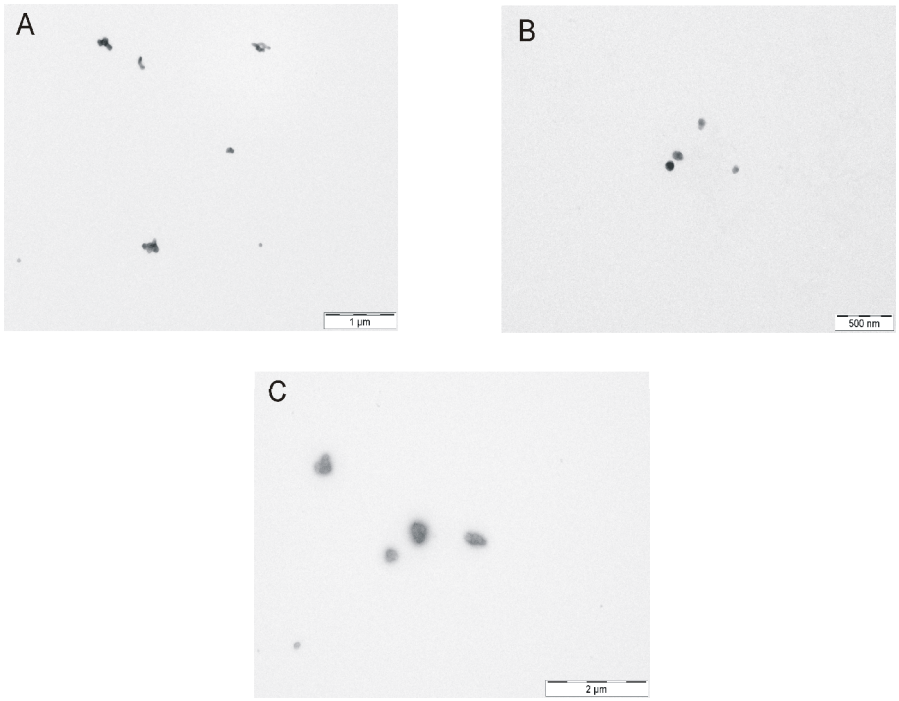

Figure 17. TEM images of DNA in the presence of (A) spermine (CE 1.8), (B) **G1** (CE 2.7), (C) **G2** (CE 2.7) – samples deposited from buffered water (pH 7.1).

In conclusion, we have presented first and second generation spermine functionalised dendrons that bind DNA with remarkably high affinities. Notably, **G2** showed salt-independent DNA binding and it was considerably more efficient than the **G1** under high-salt conditions, whilst **G1** was, in turn, significantly more effective than **G0** analogue. It can be further concluded that organised arrays of single binding units exhibit a clear effect of multivalency when prearranged on a dendritic surface and yield a high affinity DNA binding affinity that is more than the simple sum of the individual parts.

2.2. Potential as Nonviral Vectors in Gene Therapy (Article II)

In the last ten years, there has been an explosion of interest in using synthetic molecules as vectors for gene delivery.[8, 17] A range of materials such as cationic liposomes,[46, 48] polymers[125, 126] and dendrimers[127] have been utilized for this task. However, they tend to be relatively inefficient in transfection and often lack cell specific targeting. The transfection efficiency of dendrimers and dendrons is comparable to polymers and cationic lipids.[49, 128] In addition many of them are easy to modify with various fuctional groups, for example with PEG groups to reduce toxicity. Spherical PAMAM dendrimers have been studied the most because of their efficiency and commercial availability.[99] Other dendritic polyamine scaffolds have also been investigated as gene delivery systems, such as those based on a poly(propyleneimine)[104] (PPI) or dendritic L-lysine[105].

In article I we described how spermine functionalised dendrons bind DNA. This paper elucidates the relationship of these dendrons between *in vitro* transfection efficiency and toxicity. We investigated the ability of the dendrons to transfect DNA into cells (human breast carcinoma cells, MDA-MB-231, and murine myoblast cells, C2C12) as determined by the luciferase assay (Figure 18). Both cell lines were transfected *in vitro* with 1 µg of plasmid DNA per 100,000 cells. In each case, the DNA was complexed with varying

amounts of different polyamines. Gene transfection efficiency was measured as luciferase enzyme activity and normalised to total cell protein. Initially, the dendrons were investigated in their own right to determine their ability to transfect DNA into cells and allow expression of luciferase. However, no measurable transfection could be observed in any case.

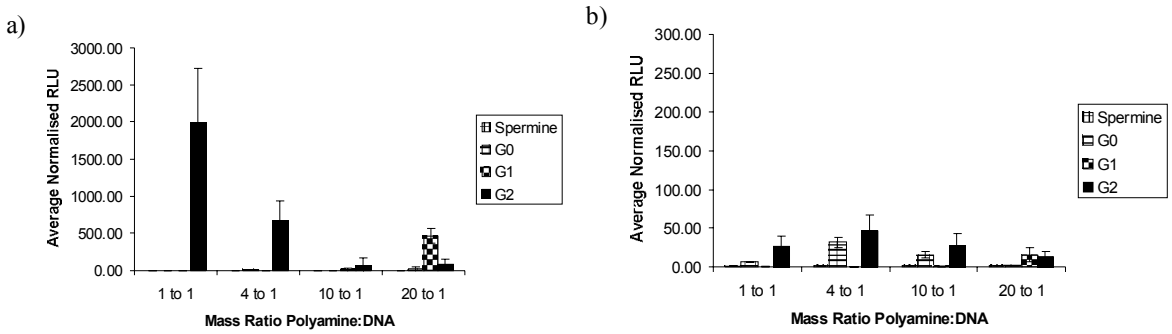

Figure 18. Transfection efficiency of spermine, the non-dendritic model (**G0**) and the asymmetric dendrons displaying spermine on the surface (**G1** and **G2**) in a) C2C12 and b) MDA-MB-231 cells. Luciferase expression was normalised by total cellular protein. (N=6, error bars represent standard deviation).

Chloroquine, a widely used 'helper molecule', was therefore added (at a final concentration of 100 μM) to aid the gene transfection. In the presence of chloroquine, measurable gene transfection was observed with some of the spermine derivatives (Figure 18). At low polyamine:DNA mass ratios (1:1 and 4:1), the second generation dendron was significantly better at transfection than the first generation analogue. However, at higher polyamine:DNA mass ratios (10:1 and 20:1) the transfection efficiency of the second generation dendron was observed to decrease sharply. Under equivalent conditions, the transfection efficiency of the first generation analogue **G1** increased dramatically. Indeed, **G1** became more effective at transfection than **G2** at high polyamine:DNA mass ratios. A similar trend was observed for both cell lines, although the transfection into murine myoblasts C2C12 was higher than that for the human breast carcinoma cells (MDA-MB-231). The non-dendritic molecules (i.e., spermine and **G0**) induced no measurable gene expression over the range of polyamine:DNA mass ratios investigated (1:1 and 4:1).

One of the major drawbacks of administrating polycationic molecules to living cells is that they have been reported to damage cell membranes as a result of the electrostatic attraction of polycations to the plasma membrane, whereas neutral and anionic polymers cause minimal damage to cellular membranes. We therefore assayed the cytotoxicity of our dendrons using an assay based on the cleavage of 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) in metabolically active cells. The same cell lines as used for transfection studies, human breast carcinoma cells (MDA-MB-231) and murine myoblasts (C2C12), were exposed to various conditions (i.e., polyamine, polyamine+DNA, polyamine+DNA+chloroquine) for 4 h, and metabolic activity was assayed 20 h later. The results of these studies are collected in Table 2.

No reduction in metabolic activity was observed when the dendritic derivatives alone were added at a concentration of 1 μg per 1000 cells. Only the 25 kDa poly(ethyleneimine) (PEI) standard reduced the metabolic activity by 30% for the C2C12 cells, indicating that our dendrons are less cytotoxic than PEI. Similarly, when a

combination of polyamine and DNA was added to the cells (0.2 μg DNA and 1 μg polyamine per 1000 cells), our dendrons (**G1** and **G2**) had no observable cytotoxicity. However, once again, the 25 kDa PEI control reduced metabolic activity – this time by 20% for the C2C12 cells.

Table 2. Cytotoxicity of spermine, the non dendritic model **(G0**), and first (**G1**) and second (**G2**) generation dendrons, as well as PEI in murine myoblast C2C12 and human breast carcinoma MDA-MB-231 cells. Cytotoxicity is reported as the effective metabolic activity, using an XTT assay. In each case, the metabolic activity of a blank sample of cells (i.e. untreated by polyamine, DNA or chloroquine) was taken as 1.000.

		metabolic activity (relative to 1.000)					
cell line	conditions	spermine	**G0**	**G1**	**G2**	PEI	CQ
C2C12	no polyamine						0.512
C2C12	polyamine alone	0.980	1.000	1.070	1.006	0.705	
C2C12	polyamine + DNA	1.659	1.032	1.032	1.015	0.808	
C2C12	polyamine + DNA + chloroquine	0.758	0.968	1.238	0.305		
MDA-MB-231	no polyamine						0.678
MDA-MB-231	polyamine alone	1.022	1.070	1.003	0.997	0.878	
MDA-MB-231	polyamine + DNA	1.099	1.063	1.011	1.034	0.954	
MDA-MB-231	polyamine + DNA + chloroquine	0.886	0.979	0.988	0.398		

a: CQ = chloroquine

Significant cytotoxicities, however, were observed in the presence of chloroquine, under experimental conditions identical to those in which transfection had been performed (i.e., 0.01 μg DNA and 0.1 μg polyamine per 1000 cells, and chloroquine at a final concentration of 100 μM). Under these conditions, chloroquine and DNA reduced metabolic activity by 50% (even in the absence of polyamines). In the presence of chloroquine, DNA and either spermine, **G0** or **G1**, the cells actually exhibited greater metabolic activities than they did in the absence of the polyamine. Indeed, in the presence of **G0** or **G1**, the metabolic activity of the cells was effectively normal. However, using chloroquine and DNA in the presence of **G2** gave rise to a marked reduction in metabolic activity (ca. 70% for C2C12 cells). In all cases, the MDA-MB-231 cells were found to be more robust, and the polyamines and chloroquine were found to have smaller effects on the metabolic activity than they do for C2C12 cells.

In summary, this paper investigated the ability of our new simple dendron structures, functionalised on the surface with multiple spermine groups, to act as vectors in gene therapy. The dendrons were unable to deliver DNA *in vitro* on their own right, however, they were capable of transfecting DNA when administered with chloroquine, which assists with escape from endocytic vesicles. Even in the presence of chloroquine the overall transfection efficiency is very low when compared to commercial transfection agents, and does not allow real applications. One of the possible reasons for low efficiency is that the DNA binding affinity of the dendrons is in fact too strong and DNA is not released in the extend needed for efficient transfection. The dendrons were non-toxic either alone, or in the presence of DNA. Conversely, when administered with DNA and chloroquine, the most highly branched dendron did exhibit varying cytotoxicity effects. It is clear that in future studies the structure of the dendrons must be modified in such way to allow efficient transfection without chloroquine. However, the current results provide encouragement that this type of building blocks, which have a relatively high affinity for DNA, will provide a useful starting point for the further synthetic development of more effective gene transfection agents.

2.3. Optically Switchable DNA Binding (Article III)

In this study we have modified our cationic multivalent dendrons presented in paper I, by introducing an *o*-nitrobenzyl link[129] between the spermine surface groups and the dendron framework. Optical irradiation can be used to cleave the ester functionality of this group from the dendritic scaffold, resulting in rapid release of the covalently bound surface groups and non-covalently bound DNA, due to dendron degradation and charge switching multivalency.

Controlling the self-assembly or function of nanoscale objects using external stimuli, such as pH, temperature, light, electric potential, or magnetic field, is an important requirement for the preparation of functional and responsive molecular machines for a wide range of potential applications. [9, 10, 80, 130, 131] Special focus has been on medicinal applications – for example, controlled drug and DNA delivery systems,[113, 132] reactivation of caged enzymes,[133] and switchable membrane proteins.[134] Light as an external stimulus enjoys a number of advantages, such as sharply defined spatiotemporal control over the responsive effect, biocompatibility and easy usage. [113, 133] DNA binding compounds that can be manipulated by light are especially interesting in DNA based computing,[4, 6, 7] 'on-chip' DNA storage[5] and non-viral gene therapy,[113, 114] because spatial and temporal control over the release event can be gained. Most of the compounds used in gene therapy bind DNA, however unpackaging of the complexes and release of the DNA is difficult to achieve if the binding is very strong. This might be responsible for low transfection efficiency, such as those results presented in publication II. DNA release is therefore of direct importance.

Recent studies on photocleavable dendrimers and dendrons, include for example self-immolative dendrimers[135], porphyrin derivatives[113] and dendrimers based on photocleavable core[136, 137] or photoactive surface.[138, 139] Given our interest in multivalent DNA recognition and transfection, we decided to explore whether our polyamine dendrons could be developed in such a way as to achieve photoresponsivity. We therefore modified our previously reported dendrons by attaching the spermine surface groups via an *o*-nitrobenzyl link (Figure 19a). The *o*-nitrobenzyl group undergoes photolytic degradation (Figure 19b) using long wavelength UV-light (λ=350 nm), thus allowing spatially and temporally controlled release of the covalently attached spermine surface groups and the non-covalently bound DNA. Once the spermine groups are cleaved from the surface of the dendron, the cationic multivalency effect is destroyed, leaving just individual spermine groups, with only weak affinity for DNA. In this way, DNA will be effectively decomplexed on photolysis. Importantly, as the surface groups are cleaved, they leave behind an anionic carboxylic acid surface that will further repel DNA and thus promote release (Figure 19c).

Polyamine dendrons with *o*-nitrobenzyl linked spermine surface groups and a benzyloxycarbonyl (Cbz) protected core were synthesised and characterised using the same methods that were used to prepare dendrons reported in publication I. The photolabile *o*-nitrobenzyl linking group (**pll**) was first connected with a peptide bond to spermine **1**, which had been appropriately protected with Boc protecting groups. The pll-spermine conjugate was subsequently coupled with the trifurcated Newkome-type branching scaffold **6** by simple esterification reaction. Standard catalyst agents and refluxing solvent were needed to form the product **18**. Crude materials were purified by using preparative GPC and silica column. Deprotection of the spermine groups using HCl

yielded highly water-soluble target dendrons **pII-G1** and **pII-G2**. The second generation dendron was synthesised in a analogous manner by esterification of pll-spermine **17** to G2-acid **9**. Model compound **pII-G0** was obtained directly from pll-spermine conjugate **17**.

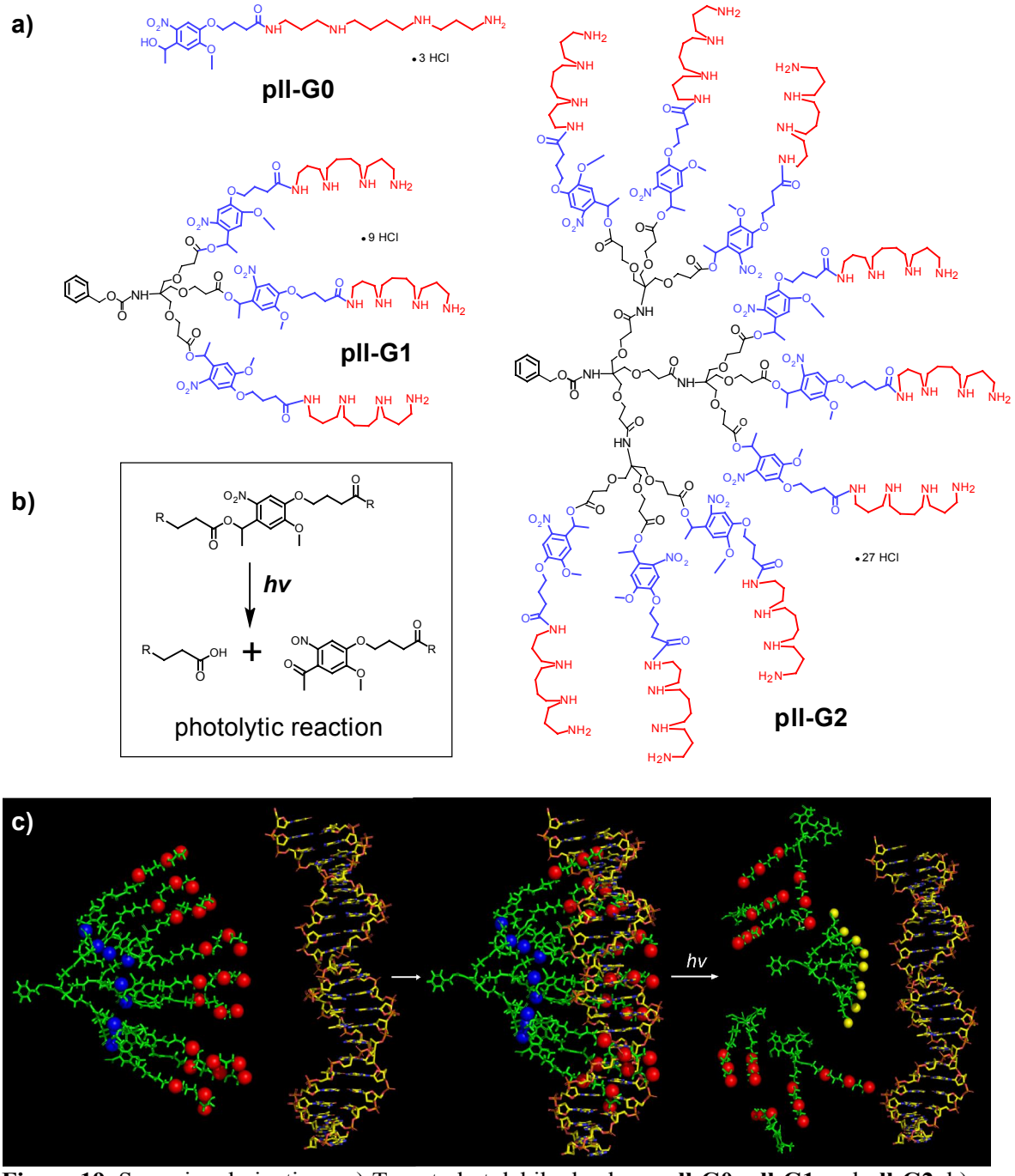

Figure 19. Spermine derivatives. a) Target photolabile dendrons **pII-G0**, **pII-G1** and **pII-G2**. b) Photolysis of **pII-G1** and **pII-G2** liberates spermine surface and exposes carboxylic acids. c) Schematic illustration of the self-assembly of multivalent dendrons and DNA, followed by the optically triggered degradation of cationic surface and release of DNA. The release is therefore due to two factors: optically triggered cleavage of the spermine chains and cationic-to-anionic charge reverse. Blue spheres: photo-cleavage sites, red spheres: cationic spermine amines, yellow spheres: anionic carboxylic acid groups exposed after photolysis.

DNA binding affinities of spermine derivatives were evaluated using an ethidium bromide displacement assay.[140] The strengths of the resulting DNA-dendron complexes were also studied by DNA relaxation using chondroitin sulfate B (csB), which is a sulfated polyanionic glycosaminoglycan known to effectively relax weak DNA-cation complexes.[141] These results are presented as a function of sulfonic acid /protonatable dendron amine (S/N) ratio.

Two different salt concentrations (9.4 mM and 150 mM NaCl) at a physiologically relevant pH value of 7.2 were again studied. Under low-salt conditions (9.4 mM NaCl) the non-dendritic compounds spermine and **pll-G0** bind to DNA, although not particularly effectively (CE_{50}= 6 and 32 respectively, Figure 20a, Table 3). The dendritic systems **pll-G1** and **pll-G2**, however, bind DNA very strongly with similar strength (CE_{50}= 0.5 and 0.4 respectively, Figure 20a, Table 3). At high salt concentration (150 mM NaCl) spermine and **pll-G0** almost completely lose their DNA binding ability (CE_{50} values >200, Figure 20b, Table 3). Conversely, **pll-G1** and **pll-G2** are only little affected by the increase of competitive Na^+ ions, due to the multivalent nature of these dendritic systems. Larger **pll-G2** binds slightly stronger than **pll-G1** (CE_{50}= 0.7 and 1.0 respectively, Figure 20b, Table 3). These CE_{50} values are in good accordance with, although slightly lower than, the values for spermine derivatives without pll-linker presented in publication I.[142]

Complex relaxation with csB at 9.4 mM NaCl salt concentration shows that **pll-G0** and spermine pack DNA into weak complexes, which are easily opened by relatively small amount of csB (Figure 20c). However, **pll-G1** and **pll-G2** form extremely strong complexes with DNA and can not be opened even with very high S/N ratios (Figure 20c). When the salt concentration is increased to 150 mM, the complexes are slightly weaker, and it is possible to open them. The **pll-G1** –DNA complex can be fully relaxed with an approximately ten-fold excess of csB, whereas **pll-G2** complexes are stronger and are relaxed at 50-fold excess (Figure 20d).

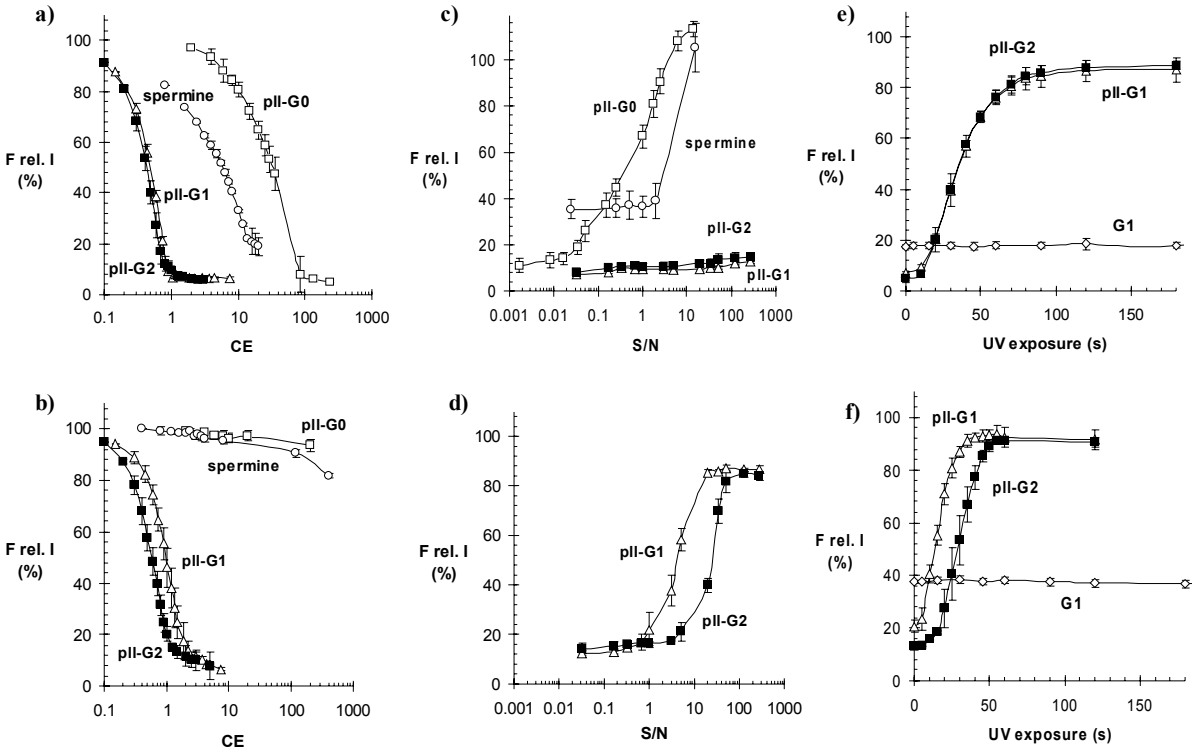

Figure 20. Titration curves for spermine, **pll-G0**, **pll-G1** or **pll-G2**. EthBr fluorescence quenching in the presence a) 9.4 mM and b) 150 mM NaCl. DNA-polycation complex relaxation with csB in the presence of c) 9.4 mM and d) 150 mM NaCl. Release of DNA from complexes by UV irradiation in the presence of e) 9.4 mM and f) 150 mM NaCl. Total added polyamine solution did not exceed 5% of the total volume; therefore corrections were not made for sample dilution. Results are the average of triplicates, error bars ± standard deviation.

Table 3. Results for spermine **pll-G0**, **pll-G1** and **pll-G2** from an ethidium bromide displacement assay.

Compound	Nominal charge	CE_{50} / 9.4 mM NaCl	CE_{50} / 150 mM NaCl
spermine	4+	6	>400
pll-G0	3+	32	>200
pll-G1	9+	0.5	1.0
pll-G2	27+	0.4	0.7

The photolytic degradation of the spermine derivatives was first studied as such, without any DNA present. Degradation was attained by irradiating an aqueous solution of **pll-G2** (Figure 21) and following the time-course of the reaction with UV-Vis spectroscopy. Irradiation of **pll-G2** compounds with UV light at 350 nm led to significant changes in the UV-Vis spectra. A decrease of absorbance at 245 nm was observed along with a clear increase at 268 nm and 349 nm – changes which typically indicate the photolytic reaction proposed in Figure 19b.[129, 136] Similar changes were observed also for **pll-G0** and **pll-G1**. Figure 21b shows that degradation of the dendritic systems reaches a plateau after ca. 200 s and **pll-G2** releases approximately three times more surface groups than **pll-G1**, which in turn releases three times more surface groups than **pll-G0**. This 9:3:1 ratio is consistent with the number of surface groups in **pll-G2**, **pll-G1** and **pll-G0** respectively. Longer irradiation times lead to further changes in the absorption spectra, for example decrease of absorption at 330-400 nm.[134]

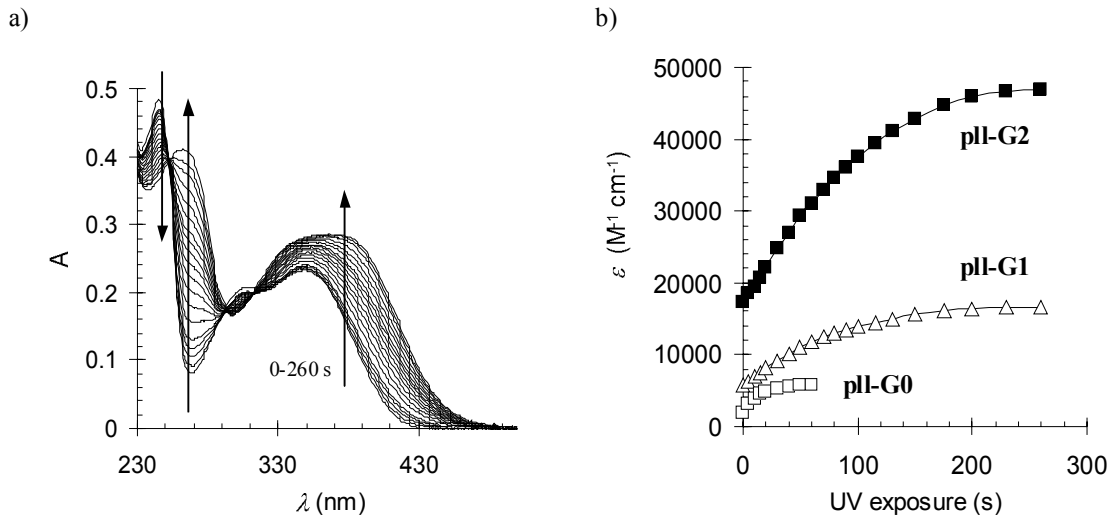

Figure 21. Photolysis of spermine derivatives. a) UV-Vis spectra of **pll-G2** after different irradiation times (0-260 s) with UV light. b) Molar absorption coefficient of **pll-G0**, **pll-G1** and **pll-G2** at 390 nm plotted against UV irradiation time indicate that the photochemical reaction takes place and **pll-G2** releases approximately three times more surface groups than **pll-G1**.

Dendron-DNA complex disassembly was then directly monitored as a function of UV irradiation time by using the ethidium bromide displacement assay. DNA was first fully complexed with the dendritic polycation (CE=2) and the resulting complexes were irradiated under UV light. EthBr fluorescence was then recorded after different time periods – if dendron disassembly occurs, the EthBr should be able to compete effectively for DNA binding with the resulting individual spermine units, and hence the fluorescence intensity of EthBr should increase. At 9.4 mM NaCl concentration EthBr fluorescence increases, indicating that both pll-dendrons release DNA after 90 s (Figure 20e). At 150 mM salt concentration **pll-G1** releases DNA rapidly after 40 s and **pll-G2** after 55 s (Figure 20f). This more rapid release might be expected as a result of the slightly weaker complexation between the dendron and DNA under the high salt conditions (particularly for **pll-G1**). Dendron **G1** (**pll-G1** without the photolabile *o*-nitrobenzyl linker, see publication I) was used as a reference under both salt concentrations and markedly no release of DNA from these complexes was observed as a result of UV irradiation. Importantly, the same trend, that at 9.4 mM NaCl concentration **pll-G1** and **pll-G2** behave similarly while at 150 mM NaCl concentration **pll-G2** binds DNA more strongly than **pll-G1**, is consistent across all three fluorescence titration methods.

DNA binding and release by pll-dendrons was confirmed by gel electrophoresis in a direct plasmid DNA (pDNA) binding assay. The photolabile dendritic constructs **pll-G1** and **pll-G2** retarded the electrophoretic mobility of DNA, whilst **pll-G0** was ineffective (Figure 22). After UV irradiation, **pll-G1** and **pll-G2** clearly released DNA, allowing its free electrophoretic mobility. Importantly, UV irradiation induced pDNA fragmentation was not observed, indicating that the structure and functionality of the pDNA is preserved under these conditions.

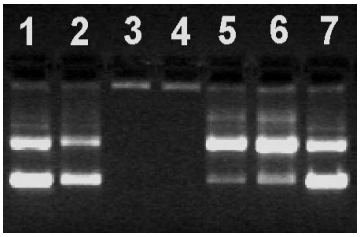

Figure 22. Gel electrophoresis of pDNA (250 ng per lane). Lane 1: pDNA. Lane 2: pDNA + **pll-G0** (CE 30) no UV. Lane 3: pDNA + **pll-G1** (CE 2) no UV. Lane 4: pDNA + **pll-G2** (CE 2) no UV. Lane 5: pDNA + **pll-G1** (CE 2) 1 min UV. Lane 6: pDNA + **pll-G2** (CE 2) 1 min UV. Lane 7: pDNA 1 min UV.

Light scattering and ζ-potential measurements studies were used to further investigate the DNA binding and releasing properties of the dendritic molecules in addition to the surface charge of the formed DNA complexes. DNA was complexed with **pll-G2** or **pll-G1** with CE 2 and the particle count-rate and ζ-potential were measured before and after one minute of UV irradiation. Before UV treatment, the observed count-rate for **pll-G2** – DNA complex was 420.9 kilo counts per second (kcps) and ζ-potential 14.7±3.4 mV, indicating the formation of a large number of positively charged particles. After UV irradiation, however, the particle count rate was found to drop off to only 8.8 kcps and the ζ-potential to –27.8±7 mV. This confirms the complex breakdown and the formation of species with a high anionic charge. The decrease in the count rate is attributed to the change in the refractive index of the dendron-DNA complexes as they undergo a transition from condensed globules to loose coils, which have a lower refractive index than dense globules. Similar behaviour was observed also by using **pll-G1** as the binding agent. Summary of the light scattering and the ζ-potential results is presented in Table 4.

Table 4. Light scattering and the ζ-potential measurement results.

	before UV-irradiation		after 1 min UV irradiation	
compound	count rate (kcps)	ζ-potential	count rate (kcps)	ζ-potential
pll-G1	380.7	18±5.8	7.9	–(19.1±4.5)
pll-G2	420.9	14.7±3.4	8.8	–(27.8±7)

In conclusion, our novel photolabile multivalent dendrons can be used for reversible DNA binding. DNA release is made possible by long-wavelength UV irradiation (λ=350 nm), which cleaves the surface groups from the dendron framework and therefore degrades and charge reverses dendron's multivalency. In particular, **pll-G1** and **pll-G2** bind DNA efficiently through complementary electrostatic interactions, but can also release their target very rapidly. Effectively, the high-affinity multivalent interactions are 'switched-off' by UV irradiation. It is therefore possible to gain spatio-temporal control over DNA binding and release, making these dendrons very promising for detailed applications in nanobiotechnology.

3. HIGH AFFINITY ADHESION OF PROTEINS TO DNA

Nature has evolved a vast repository of proteins and enzymes to carry out a wide range of sophisticated tasks, which have been extensively exploited by biotechnology and medicine.[143] Such properties are, however, only as good as conferred by nature, and efforts to improve or to alter the biological properties of proteins have been made in various ways.[144] One of these methods is to modify the protein by attaching a polymer chain covalently onto its surface.[126] Examples of such modifications have yielded high-affinity binding to biomolecules, tissue or intracellular targeting by multivalent binding to cell surface receptors,[145] prolonged circulation lifetime,[146-150] thermal switching of enzyme activity,[151] and size-dependent binding.[152] However, approaches that would mimic the DNA binding properties of natural proteins[153] have previously received little attention.

DNA binding and transfection ability of polyamine dendrons was studied and demonstrated in articles I-III. In this chapter, the data of the last two publications (IV and V) is presented, where we describe how the dendrons can be attached onto protein surfaces and how they function. It is demonstrated that the DNA binding ability of the dendrons can change the biological properties of proteins, enabling them to self-assemble with DNA.

3.1. Synthesis of Protein-Dendron Conjugates for DNA Binding (Articles IV and V)

Methods to prepare well-defined protein-polymer conjugates[144, 146, 154-156] can be divided into two different classes: "*grafting to*"[157-161] and "*grafting from*[162-164]: The former way utilises a protein-reactive polymer, which can be attached onto reactive groups on protein surface and the latter initiation sites attached on the protein surface where polymerisation can take place directly. Alternatively modification can be achieved by cofactor reconstitution.[165-167] Most studies concentrate on linear polymers, but a drawback to their use is that they induce a degree of heterogeneity in the form of both the attached polymer and often the protein attachment sites. Dendrons, however, are not afflicted by these problems. Conversely, only relatively few studies on protein-dendron conjugates exist, including for example, protein with dendritic bisphosphonic acid,[168] anionic myoglobin derivatives,[159, 166, 167] dendrons with multiple proteins on surface,[169] synthetic glycoproteins[170], insulin modified with sialic acid[171] and PAMAM-biotin conjugates.[172]

We chose to use the maleimido chemistry to target a single free cysteine residue on protein surface. *N*-maleimido group is well known to react very selectively with free sulfhydryl groups in neutral aqueous solutions and ambient temperature. *N*-maleimido cored first and second generation dendrons were prepared from the synthesis intermediates **7** and **10**, used in the preparation of polyamine dendrons presented in publication I. Deprotection of the Cbz-protected amino core using catalytic hydrogenation yielded the free amines at the focal point (compounds **11** and **14)**, which were subsequently reacted with an excess of 3-maleimidopropionic acid to afford cysteine reactive dendrons **12** and **15**. Deprotection of the spermine groups using HCl then yielded highly water-soluble target dendrons **13** and **16** with an intact *N*-maleimido group at the core. However it is noteworthy that while the synthesis of dendrons might seem to be a simple process, it is still relatively difficult to produce bulk quantities of high generation dendrimers, because of the multiple synthesis and purification steps.

Ideal proteins for precise conjugation should contain only one reactive sulfhydryl group, although methods to modify native disulfide-bridged cysteines using a thiol-specific, cross-functionalised monosulfone have also been studied.[173-175] Precise conjugation is essential if the protein functionality is to preserved; conjugation of a large molecule too close to proteins active part could alter or hamper its functionality dramatically. Free cysteines that are reactive because they do not take part in disulfide bridge formation are rare in proteins: for example a globular Bovine Serum Albumin (**BSA**) contains naturally only one reactive cysteine (Cys-34). Furthermore, approximately 50% of these cysteine residues are oxidised and thus unavailable for conjugation.[176] Nonetheless, **BSA** was chosen as our large (66.4 kDa, 607 amino acids) model protein. Serum albumin is the most abundant plasma protein in mammals and functions as non-specific carrier for several hydrophobic compounds such as fatty acids and steroid hormones, carrier for unconjugated bilirubin and calcium ion binder. It also partly maintains the osmotic pressure in blood plasma by preventing water from crossing capillary walls into tissue. **BSA** structure consists of three homologous looping domains held together by 17 disulphide bonds (Figure 23). Domain structures are highly alpha-helical and lack beta-sheets. **BSA** is readily available commercially and exhibits other potential advantages, such as long circulation time and low toxicity. Indeed, a commercial drug formulation Abraxane incorporates albumin to improve the solubility and reduce the toxicity of paclitaxel.[177]

Class II hydrophobin (HFBI) from *Trichoderma reesei*[178-183] was chosen as another protein for the protein-dendron conjugation reactions.[184] In common globular proteins the hydrophobic residues are buried inside the protein, but HFBI has a very unusual amphiphilic structure where approximately half of its hydrophobic amino acid side chains form a hydrophobic patch exposed to the protein surface. HFBI can therefore be regarded as a mesoscale surfactant protein. Mesoscale surfactants are thought to have very interesting properties in hydrophobic assembly.[185, 186] The HFBI fold forms two β-harpins linked by a short alpha-helix resulting in an antiparallel β-barrel structure (Figure 23). The β-barrel is further stabilised by four cross linking disulfide bridges, which are buried inside the protein and make the fold very compact and relatively hard to denaturate. Due to its surface activity, HFBI is known to form various structures through spontaneous self-assemby. Indeed hydrophobin films with a self-assembled hexagonally ordered structure are now well characterised.[187-189] The natural structure of HFBI does not provide a single free cysteine residue, therefore site-directed mutagenesis was used to construct a protein variant of HFBI with a free sulfhydryl group for site-specific conjugation. The protein variant, termed NCys-HFBI, was produced in its homologous production host *Trichoderma reesei* and purified from the fermentation biomass yielding partially oxidised covalent dimer of NCys-HFBI.[179] After purification, disulfide bridged NCys-HFBI dimers were reduced to monomers (**HFBI)** with dithiothreitol.

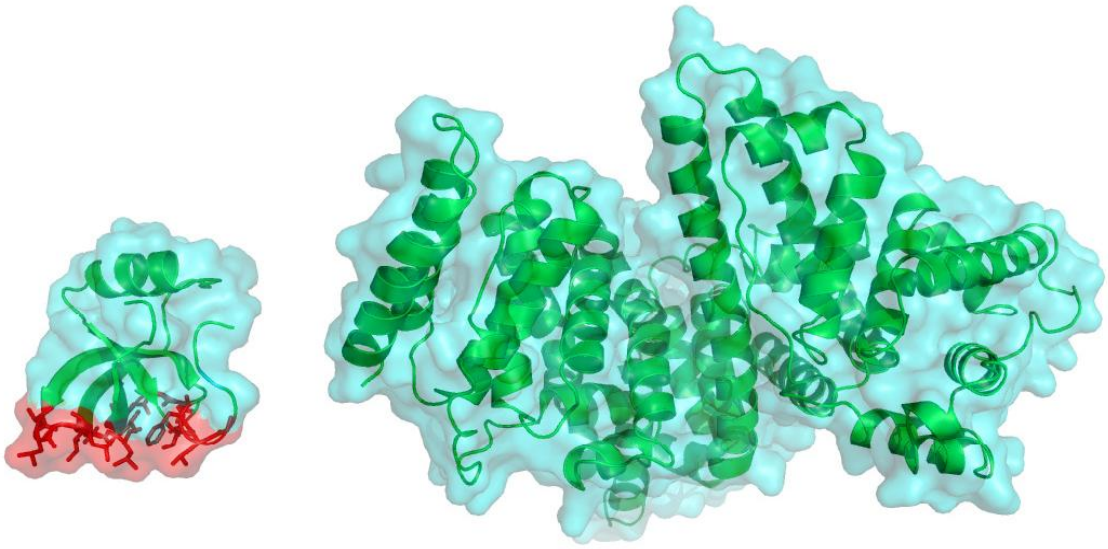

Figure 23. Structure of HFBI (left) and **BSA** (right). The conserved side chains of the hydrophobic patch of HFBI are shown in red.

The reduced **HFBI** was conjugated to the first and second generation dendrons in buffered aqueous solution at neutral pH using an excess of dendron (Figure 24). Conjugation to **BSA** was done with similar procedure, however **BSA** was not reacted with dithiothreitol prior to conjugation. To avoid confusion, it must be noted that **BSA** contains a 24 amino acid signal and propeptide sequence not observed in the final translated and transported protein but is present in the gene. Therefore the free cysteine is the 58th amino acid in the sequences found from databases. The 1,4-conjugate addition reaction between *N*-maleimido group and free cysteine sulfhydryl group was allowed to proceed at least overnight to ensure maximal conversion.

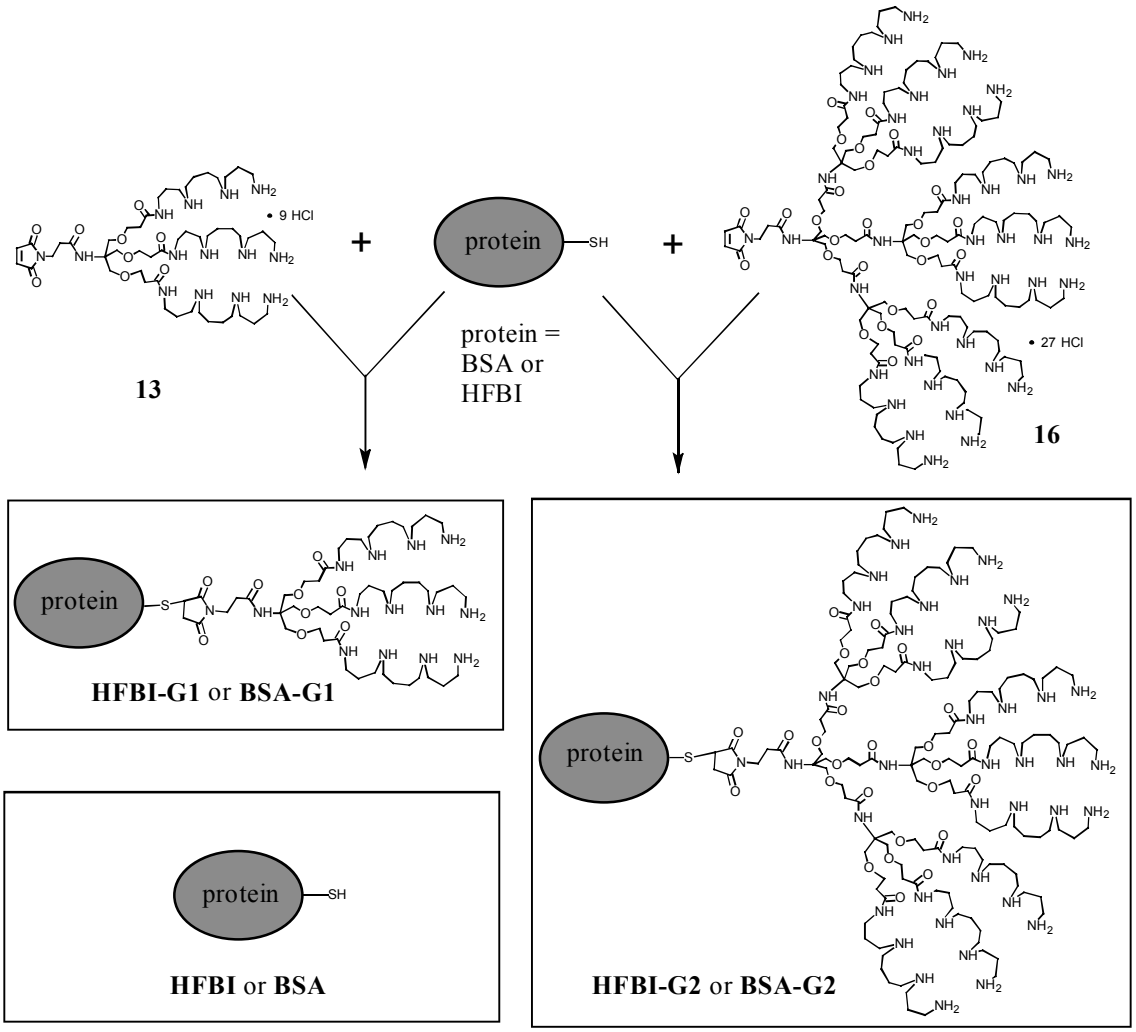

Figure 24. Target dendrons for protein modification (**13** and **16**) and preparation of protein-dendron conjugates (**BSA-G1**, **BSA-G2**, **HFBI-G1** and **HFBI-G2**). Reaction conditions: H_2O, pH 7.

Analytical data for **BSA**, **HFBI** and the protein dendron conjugates is presented in Figure 25 and Table 5. Purification by semi-preparative HPLC allowed the separation of free protein form the protein dendron conjugates. Separation efficiency was less effective for **BSA** conjugates than for **HFBI** conjugates, probably because the attached dendron does not alter the retention of a large hydrophilic protein as much as a small amphiphilic one. Even so, the separation efficiency was enough to recover pure material in all cases. Peaks were fractionated, pooled and finally lyophilised to yield the products as white solids. High purity after pooling was confirmed by analytical HPLC, which shows a single symmetric peak for all purified compounds. The elution times within the protein series gradually decrease as the growing size of the dendron increases water solubility and therefore hinders retention. For both proteins smaller retention volume was observed with dendron attachment and increasing dendritic generation as would be expected because of the high hydrophilicity of the dendrons. Overall larger retention volume of **HFBI** conjugates is consistent with their higher hydrophobicity when compared to **BSA** conjugates. Analysis by matrix-assisted laser desorption ionisation – time of flight (MALDI-TOF) mass spectrometry shows clear signals with good accordance to the calculated mass of the conjugates, confirming the covalent structure of the target molecules (Table 5, Figure 25b). CD measurements were used to confirm that the protein

structure is not detectably changed by the attached dendron (Figure 25c). CD spectra for **BSA** based compounds are consistent with the high content of helical secondary structure. The spectra for **HFBI** and its conjugates indicate rich random coil and β-sheet content, also consistent with the protein native structure. Details of the analytical data are presented in Table 5. The results show that this is a convenient method to prepare exact one-to-one protein-dendron conjugates in good yield (~80% for HFBI and ~50% for BSA) and could also be applicable to other functionalised dendrons.

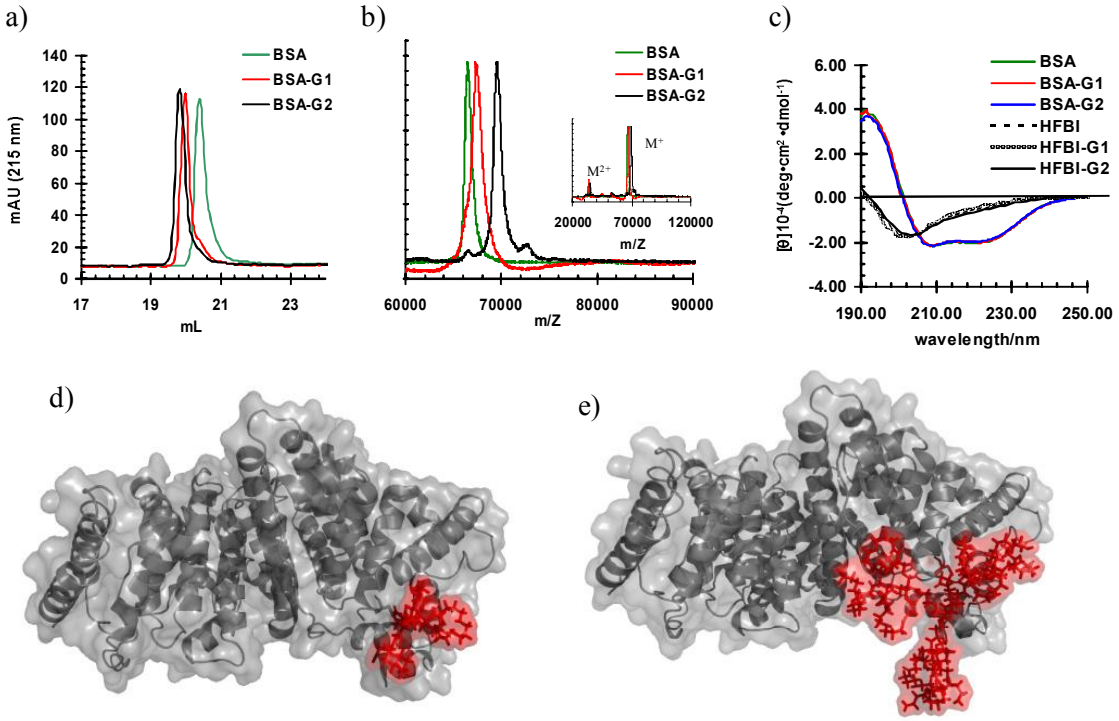

Figure 25. Analytical data for protein-dendron conjugates. a) Analytical HPLC chromatogram of the purified **BSA**, **BSA-G1** and **BSA-G2** showing a decreasing retention volume with increasing dendritic generation, see Table 5 for values. b) MALDI-TOF spectra of purified **BSA**, **BSA-G1** and **BSA-G2** showing increasing mass with increasing dendritic generation, see Table 5 for values. c) CD-spectra for all studied proteins and their dendron conjugates confirming that the protein structure is not detectably changed by the attached dendron. A schematic computer generated model of d) **BSA-G1** and e) **BSA-G2**. Cys-34 and the attached dendron are shown in red.

Table 5. Analytical data for protein-dendron conjugates.

Compound	Calc. mass / g mol^{-1}	MALDI-TOF / $m\,z^{-1}$	Retention volume / mL (semi-preparative HPLC)	Conjugation yield (%)[a]	Retention volume / mL (analytical HPLC)
BSA	66 430.3[190]	66 444.3	76.96	-	20.33
BSA-G1	67 471.7	67 404.1	75.70	53	20.00
BSA-G2	69 535.6	69 552.5	72.71	48	19.82
HFBI	8 676.7	8 676.5	107.45	-	23.86
HFBI-G1	9 718.1	9 722.4	98.49	79	22.87
HFBI-G2	11 782.0	11 782.8	91.97	83	21.13

[a] Approximated from peak heights (semi-preparative HPLC).

In summary, we have described *N*-maleimido cored dendrons that selectively react via 1,4-conjugate addition with a single free thiol group on the protein surface – Cys-34 of

Bovine Serum Albumin (BSA) or genetically engineered cysteine mutant of Class II hydrophobin (HFBI). Because the location of the thiol group is known exactly, the resulting one-to-one protein-polymer conjugates are extremely well defined – the number of dendrons and their attachment site on the protein surface is precisely known. The conjugation reaction can be conducted in mild aqueous solutions (pH 7.2-7.4) and ambient temperature resulting in BSA and HFBI-dendron conjugates in yields of at least 48% and 79% respectively.

3.2. Functionality of Protein-Dendron conjugates (Articles IV and V)

After establishing robust synthetic and purification methods to prepare sufficient amounts of protein-dendron conjugates, a series of different methods were utilised in order to asses the various functional properties of the protein dendron conjugates.

3.2.1. DNA Binding

In the EthBr displacement assay both unmodified proteins, **BSA** and **HFBI**, were unable to bind DNA. This was expected as commonly proteins that do not have DNA binding motifs or significant surface positive charge do not bind DNA. No interaction with DNA was observed even with high protein concentrations (Figure 26a,d, Table 6). However, the protein-dendron conjugates showed significantly enhanced DNA binding. Under low-salt conditions (9.4 mM NaCl) **HFBI-G1** and **HFBI-G2** bind DNA very strongly and with similar affinity (CE_{50}=0.6, Figure 26d, Table 6). **BSA-G2** also binds strongly, however with slightly lower affinity (CE_{50}=1.0, Figure 26a, Table 6). **BSA-G1** exhibits lower binding affinity (CE_{50}= 3.5, Figure 26a, Table 6) when compared to other protein-dendron conjugates. The lower binding affinity for this dendron conjugated **BSA** might be expected because the rather small dendron (ca. 1 kDa) must adhere a much larger **BSA** protein (ca. 66 kDa) to DNA. At physiological salt concentration (150 mM) **BSA-G1** and **HFBI-G1** both exhibit weaker binding than at low salt concentration (CE_{50}=6.3 and 0.9 respectively, Figure 26b,e, Table 6). The binding affinity of **BSA-G1** is affected the most. It also interesting to notice that **BSA-G1** binds DNA with noticeably lower affinity than **G1** (see publication I) alone – this is presumably a consequence of the entropic cost of binding the high molecular mass **BSA** protein to the DNA. Nonetheless, the measured binding value is surprisingly strong. On the contrary **BSA-G2** and **HFBI-G2** are little affected by the increase in salt concentration and they bind DNA with extremely strong affinities (CE_{50}=0.6 and 0.5 respectively, Figure 26b,e, Table 6) as a consequence of their multivalent nature.

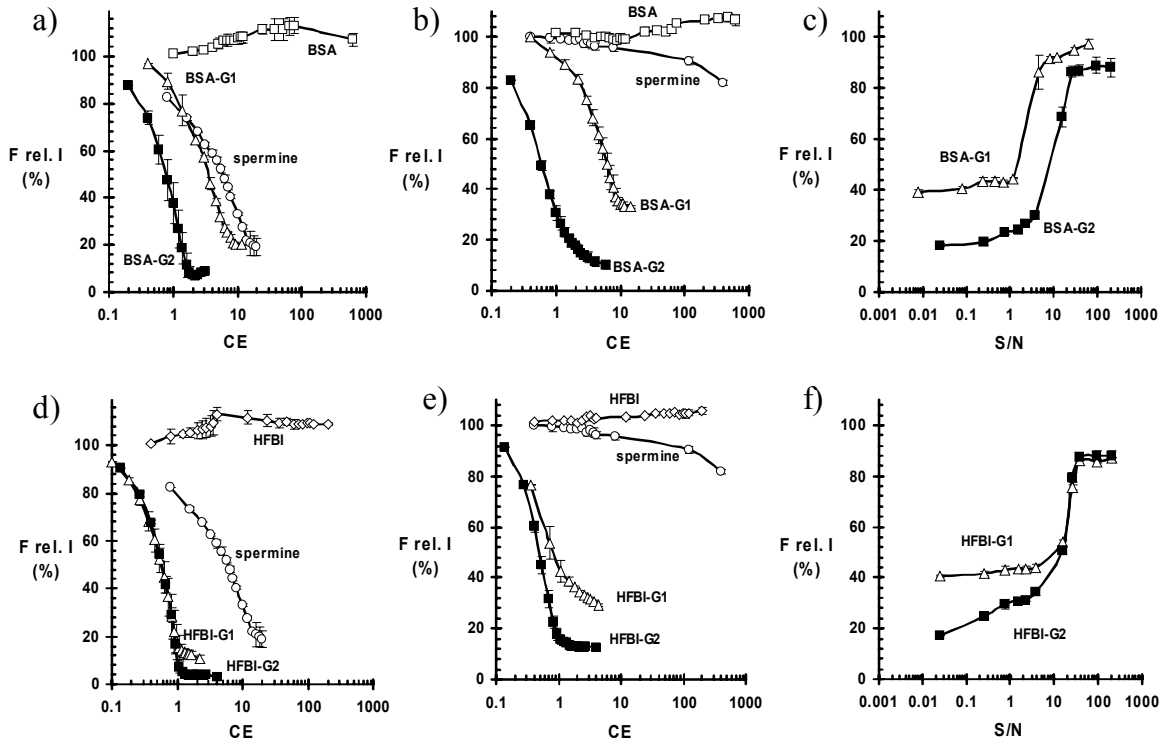

Figure 26. Ethidium bromide displacement assay curves for spermine, **BSA**, **BSA-G1**, **BSA-G2**, **HFBI**, **HFBI-G1** or **HFBI-G2** in a solution of 1 μM DNA and 1.26 μM ethidium bromide in buffered water (pH 7.2). EthBr fluorescence quenching in the presence a,d) 9.4 mM and b,e) 150 mM NaCl. DNA-polycation complex relaxation with csB in the presence of c,f) 150 mM NaCl. Results are the average of triplicates, error bars ± standard deviation.

Complex strength evaluation using csB as relaxing agent shows that **BSA-G2** packs DNA into a strong complex, which can be fully opened only with an excess of csB (S/N ratio >25, Figure 26c). Complexes formed with **BSA-G1** are significantly weaker and can be opened with relatively small S/N ratio (S/N ratio ca. 5, Figure 26c). Smaller HFBI-dendron conjugates are able to pack DNA even more strongly when compared to **BSA** conjugates. Both **HFBI-G1** and **HFBI-G2** pack DNA into strong complexes with similar strength. Both complexes can only be fully relaxed at S/N ratios of approximately >30 (Figure 26f).

Table 6. Results for spermine **BSA**, **BSA-G1**, **BSA-G2**, **HFBI**, **HFBI-G1** and **HFBI-G2** from an ethidium bromide displacement assay.[a]

Compound	Nominal charge	Calculated FW [g/mol]	CE_{50} / 9.4 mM NaCl	CE_{50} / 150 mM NaCl
spermine	4+	362.5	6.0	>400
BSA	(9+)[b]	66 430.3[190]	>400	>400
BSA-G1	9+	67471.7	3.5	6.3
BSA-G2	27+	69 535.6	1.0	0.6
HFBI	(4+)[c]	8676.7	>200	>200
HFBI-G1	9+	9718.1	0.6	0.9
HFBI-G2	27+	11782.0	0.6	0.5

[a] Total added polyamine solution did not exceed 5% of the total volume; therefore corrections were not made for sample dilution. Results are an average of three titrations. [b] BSA has an overall negative surface charge (pI<6) at neutral pH, however nine positive charges were assumed for comparison. [c] According to protein amino acid sequence and the number of protonable side chains, four positive charges were assumed.

Taken the results together, they demonstrate that a functional DNA binding dendron can impart its properties onto the protein to which it is attached. This method enables us to convey DNA affinity to proteins that do not have a natural DNA binding affinity. The binding values measured for the protein-dendron conjugates are, especially for **BSA** conjugates, surprisingly strong and in general agreement with those measured for the dendrons alone (see publication I). The binding affinity of **BSA-G1** is relatively strong and shows that even a rather small (ca. 1 kDa) **G1** dendron can convey reasonable binding affinity even to a much larger protein (ca. 66 kDa). This kind of behavior much resembles the binding of natural proteins that rely on DNA binding domains.[153] Dendrons attached to the protein surface can therefore be described as synthetic DNA binding domains.

3.2.2. Surface Self-Assembly

The amphiphilicity of the HFBI-dendron conjugates was first studied on solid surface. Considering the possible applications of these conjugates in gene therapy, the hydrophobicity of the carrier is known to strongly influence the DNA transport through cellular membranes into cells[107] and is therefore important to characterise. Quartz crystal microbalance (QCM) was used to study the surface adhesion and binding properties of **HFBI**-dendron conjugates. QCM can measure small changes in mass on a quartz crystal resonator in real-time. During measurement two types of information are given. The change in crystal's resonance frequency (Δf) is directly proportional to the absorbed mass on the crystal, and the dissipation (D) change gives information about the decay of the resonance signal, which is dependent on the structure of the absorbed layer. Usually high dissipation energy indicates flexible or loose structure.

One of the remarkable properties of hydrophobins is their ability to form self-assembled films on hydrophobic surfaces or air-water interface.[187, 191] These features have been demonstrated previously for various different types of hydrophobins,[191] however we wanted to demonstrate the same effect also with protein-dendron conjugates. The **HFBI** and **HFBI-G2** were immobilised on hydrophobic polystyrene coated crystals. Figure 27 shows the adsorbtion of both compounds at concentration of 50 μg/mL. Both compounds adsorb rapidly in a similar manner, reaching the maximum level almost instantly, as would be expected for amphiphilic proteins. However, **HFBI-G2** induces larger dissipation

compared to **HFBI** alone and is attributed to the fact that the flexible second generation dendron attached to the protein induces flexibility also to the conjugate (Figure 27). Washing with buffered water did not reduce the amount of surface bound compounds. After immobilisation and washing of the functionalised sensor crystals, their interaction with DNA was examined. DNA was injected in the same buffer system at 0.2 mM nucleotide concentration. As expected, **HFBI** does not show any interaction with DNA seeing that no shift in frequency or dissipation energy was observed. **HFBI-G2**, however, showed a clear shift in frequency, indicating DNA binding. Also the clear reduction in dissipation energy indicates that the dendron becomes more rigid as a result of DNA binding along the surface.

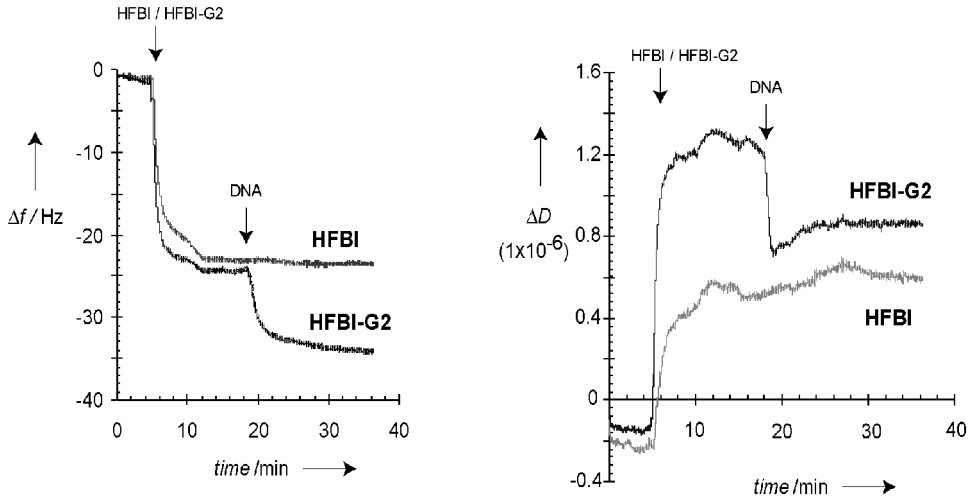

Figure 27. Surface adhesion and DNA binding properties of **HFBI** and **HFBI-G2** as measured by the change in resonance frequency (a) and dissipation energy (b). **HFBI** or **HFBI-G2** was added after 3 min, DNA was added after 16 min.

The surface-activities of **HFBI-G1** and **HFBI-G2** on air water interface were studied by compressing Langmuir films (Figure 28a). The unmodified **HFBI** isotherm shows a steep liquid-condensed behaviour and a collapse at ca. 35 mN/m. **HFBI-G1** shows a rapid rise of the surface pressure at mean molecular area (Mma) ca. 60 $Å^2$ and a collapse point at 60 mN/m, whereas measured Mma value of **HFBI-G2** is shifted even lower to ca. 10 $Å^2$ and a collapse point at 56 mN/m. These results further verify the amphiphilicity of the HFBI-conjugates and agreeably show dendritic effect to the film formation, where increasing the dendritic generation on the protein surface makes it more soluble in the subphase and therefore sifts the Mma to lower $Å^2$ values because an increasing amount of the material is lost into the subphase.

Atomic force microscope (AFM) was used to directly image and assess the crystallinity of the protein-dendron conjugate films. The films were prepared using a Langmuir trough compression and then deposited onto a graphite substrate, after which they were dried and imaged with AFM. The AFM images reveal that dendron modified hydrophobins can form stable films on air-water interface and that the films have a regular hexagonal-like structure with the dimension of a few nanometers (Figure 28b,c). The same kind of structure was observed for both **HFBI-G1** and **HFBI-G2** films. Analysing the structured parts of the surfaces using Fourier transform yielded 2D crystal unit cells of a=5.9 nm,

b=5.4 nm, γ=119° for **HFBI-G1** and a=5.3, b=4.9, γ=115° for **HFBI-G2**, indicating a close to hexagonal packing in both cases (Figure 28b,c inset).

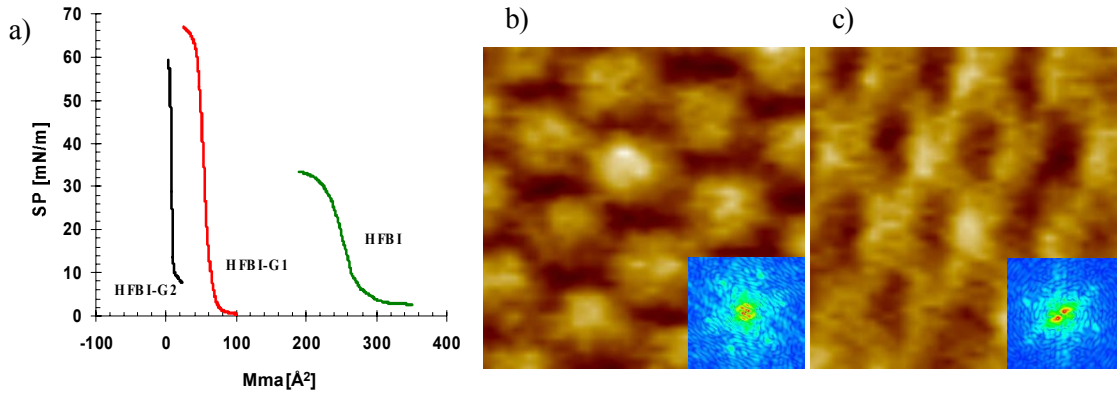

Figure 28. a) Surface pressure - area isotherms obtained by compressing **HFBI-G1** or **HFBI-G2** Langmuir films. The **HFBI** isotherm is plotted as a reference. b,c) Correlation averaged AFM topography image of b) **HFBI-G1** and c) **HFBI-G2** Langmuir film showing a regular ordered hexagonal patterns of objects. Image size is 19 nm x 19 nm. Insets: Fast Fourier transforms of single crystalline areas of **HFBI-G1** or **HFBI-G2** film.

In summary, these results demonstrate that **HFBI**-dendron conjugates can adhere on hydrophobic surfaces and bind DNA. Behaviour of the conjugates on air-water interface show how the surface-activity of the **HFBI**-dendron conjugates is dependent on the attached dendron. In addition, AFM images confirm that the conjugates can self-assemble into a similar hexagonal array as **HFBI** alone.

3.2.3. Cytotoxicity and gene transfection

Cytotoxicity is an important feature in non-viral gene therapy, as ideal vectors should exhibit low or nonexistent toxicity. Proteins have diverse effects on cellular metabolism, however for example **HFBI** and **BSA** in particular are well known for their safety. Polycationic compounds, however, are known to damage cell membranes as a result of their electrostatic interactions with the plasma membrane.[132] With this potential drawback in mind, cytotoxicity of our protein-dendron conjugates towards fibroblasts cells (CV1-P) was assessed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. None of the studied compounds showed any toxicity at CE ratios 0.125-4. However, **HFBI-G1** and **HFBI-G2** were observed to be slightly cytotoxic at high CE ratios, where relative cell viability decreased markedly (Figure 29a). **HFBI** did not reduce cell viability at any CE ratio. At CE 16 **HFBI-G1** decreased relative cell viability to ca. 62% and **HFBI-G2** to ca 50% (Figure 29a). **BSA** and its dendron conjugates did not indicate any toxicity.

Recent studies in protein-polymer conjugates have shown that they can be used as non-viral vectors in gene therapy. For example conjugates containing a cationic polymer such as polyethyleneimine (PEI) or poly(L-lysine) and an immunoglobulin, have been developed.[118, 120, 121] In these conjugates the cationic polymer is used to bind and compact DNA and the antibody being selected to facilitate receptor mediated gene delivery into various cell types.[122] Our approach is similar; the cationic dendron is used to bind DNA and the protein functionality is used to increase penetration through cellular membranes. We investigated the protein-dendron conjugate mediated gene delivery into CV1-P cells

with varying CE ratios of (Figure 29b). Gene transfection efficiency was measured as β-galactosidase expression. PEI 25k and plain pDNA were used as positive and negative controls respectively. We observed clearly enhanced transfection only for **HFBI-G2** with high charge excess ratio (≥4), while all the other protein-dendron conjugates were unable to mediate efficient transfection. Optimal transfection efficiency was achieved at CE 4 and notably, no cytotoxicity was observed with this CE ratio. We suspect that this increase in the transfection efficiency is due to the protein amphiphilicity, because the results presented in Publication II indicated that the dendrons alone are relatively ineffective transfection agents and **BSA** and its dendron conjugates did not induce measurable β-galactosidase activity. Clearly this is not due to a weak or different DNA binding mechanism of **BSA-G2**, because both **HFBI-G2** and **BSA-G2** bind DNA in a similar manner. However, higher surface-activity does not alone increase transfection because **HFBI-G1** is not efficient even though it is more surface-active than **HFBI-G2**. There have been no previous studies on the interactions between hydrophobins and biological membranes, but the current results indicate that these interactions would make an interesting future study.

Taken the results together, we have demonstrated that HFBI modified with a DNA binding dendron, functions as a cationic surfactant capable of delivering DNA across biological membrane and is not markedly cytotoxic at low CE ratios. It must be noted that the overall transfection efficiency of **HFBI-G2** is low when compared to PEI 25k, which induced over 20-fold higher β-galactosidase activity. However, this is significantly better than the previously reported behavior of simple **G2** dendron as a transfection agent (see Publication II).

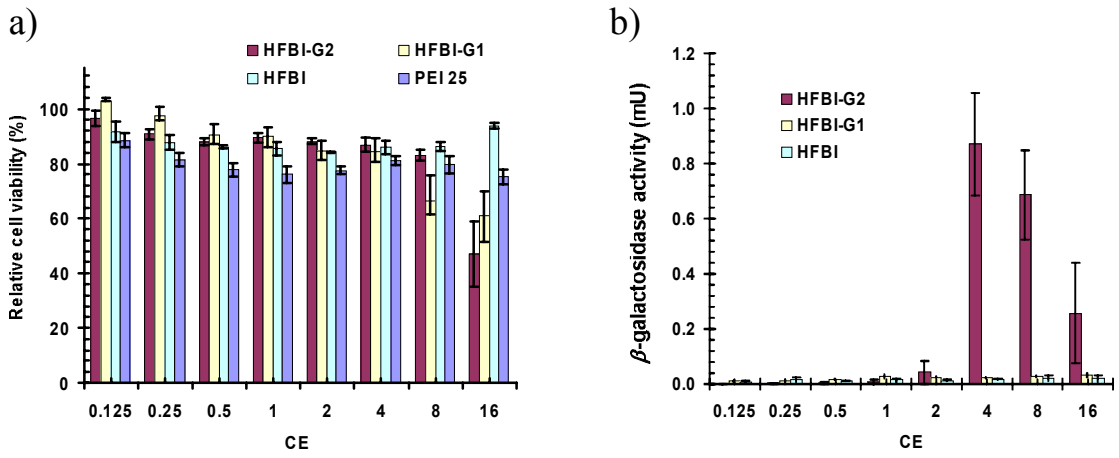

Figure 29. a) Cytotoxicity of **HFBI**, **HFBI-G1**, **HFBI-G2** and PEI 25k in kidney fibroblast cells (CV1-P) reported as relative cell viability (%). b) Transfection efficiency of **HFBI**, **HFBI-G1** or **HFBI-G2** in CV1-P cells given as mU of β-galactosidase activity. Results are the average of triplicates, error bars represent the standard deviation.

4. CONCLUSION

This Thesis presents novel dendritic derivatives for high-affinity DNA binding. The dendrons consist of Newkome-type polyether framework and multiple spermine units, nature's own DNA binder, on the surface of a dendritic scaffold. The dendrons interact with DNA in generation dependent (**G2**>**G1**>**G0**) manner with the more highly branched dendrons being the strongest DNA binders probably due to the chelate and statistical effect of multivalency (Figure 5, page 8). Importantly, the linear **G0** analogue was unable to bind DNA at physiological salt concentration.

Gene transfection efficiency of these dendrons remained very poor, even when administrated with chloroquine, which assists escape from endocytic vesicles. One possible reason for low transfection efficiency is that the interaction between the dendron and the DNA is in fact too strong and therefore DNA is not released. The dendrons, however, were not markedly toxic either alone or in the presence of DNA.

In order to address the DNA release problem, optically triggered release of DNA from the dendrons was made possible by attaching the surface spermine groups by *o*-nitrobenzyl to dendron frame. Upon photolysis the surface groups are cleaved leaving behind an anionic carboxylic acid surface and only individual spermine groups, which were previously found not to be able to bind DNA. Due to this degradation and charge switching of multivalency, the DNA is rapidly released.

DNA binding dendrons can also be attached onto protein surfaces by employing maleimido chemistry to yield precisely defined protein-polymer conjugates where the number of dendrons and their attachment site are precisely known. It is therefore possible to convey DNA affinity to proteins that do not have natural DNA binding ability. Importantly the DNA binding ability of the second generation dendron is not affected even when it is bound on the surface of a large biomolecule. The protein part in the conjugate can also play an active role by for example promoting surface adhesion or transfection efficiency.

We expect that the functional dendrons and the union between proteins and multivalent synthetic compounds open a route to novel applications in gene protection and delivery. It is further possible to 'fine-tune' the functionality of the dendron periphery by means of organic synthesis and to further control the interactions between molecules and the self-assembly of nanoscale bioconjugates in more general way.

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ABSTRACTS OF PUBLICATIONS I-V

I. High-affinity binding between nanoscale objects is an essential prerequisite for "bottom-up" fabrication. In recent years, interest has focused on the use of dendritic macromolecules as supramolecular nanoscale building blocks. The branched superstructure of dendrons and dendrimers offers specific advantages, for example, enhancement of weak binding by using multivalent arrays of recognition units on the dendritic surface. This multivalency principle, in which organized arrays amplify the strength of a weak binding process, such as the binding of saccharides to proteins on cell surfaces, is now well established. We are interested in optimising DNA binding and developing low-molecular-mass dendrons with very high affinities for DNA—such systems are particularly useful for DNA encapsulation and protection. Herein, we report on multivalent dendritic spermine constructs with well-defined molecular structures and extremely high, salt-independent binding affinities for DNA. These monodisperse systems enable an understanding of structure–activity relationships and, in addition, have a greater chance of being licensed for therapeutic applications in the longer term.

II. This paper investigates a series of dendrons based on the Newkome dendritic scaffold that displays a naturally occurring polyamine (spermine) on their surface. These dendrons have previously been shown to interact with DNA in a generation dependent manner with the more highly branched dendrons exhibiting a strong multivalency effect for the spermine surface groups. In this paper, we investigate the ability of these dendrons to transfect DNA into cells (human breast carcinoma cells, MDA-MB-231, and murine myoblast cells, C2C12) as determined by the luciferase assay. Although the dendrons are unable to transfect DNA in their own right, they are capable of delivering DNA in vitro when administered with chloroquine, which assists with escape from endocytic vesicles. The cytotoxicity of the dendrons was determined using the XTT assay, and it was shown that the dendrons were nontoxic either alone or in the presence of DNA. However, when administered with DNA and chloroquine, the most highly branched dendron did exhibit some cytotoxicity. This paper elucidates the relationship between in vitro transfection efficiency and toxicity. While transfection efficiencies are modest, the low toxicity of the dendrons, both in their own right, and in the presence of DNA, provides encouragement that this type of building block, which has a relatively high affinity for DNA, will provide a useful starting point for the further synthetic development of more effective gene transfection agents.

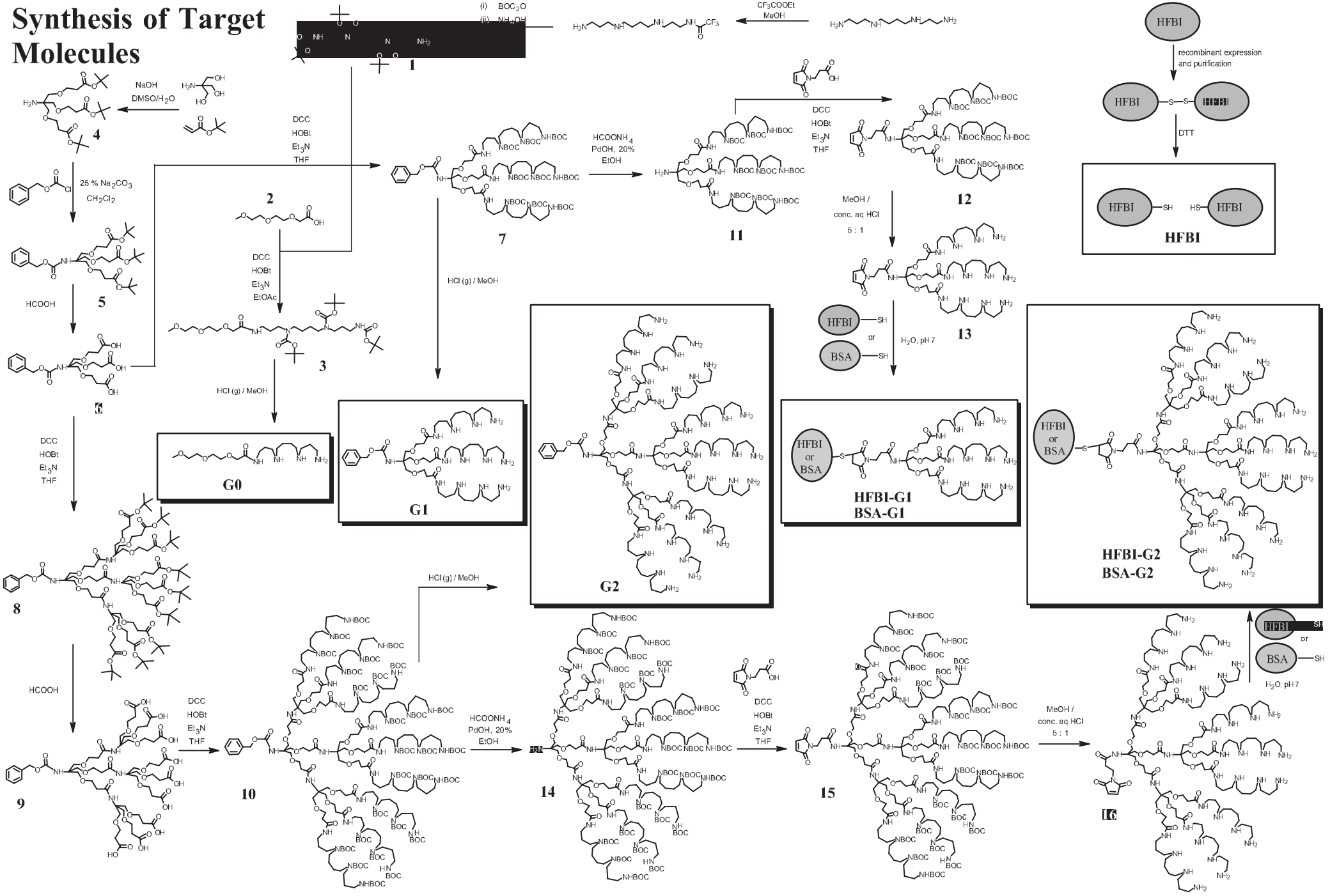
III. Multivalent binding between nanoscale objects has recently emerged as one of the most powerful methodologies for the assembly of functional supramolecular materials with applications in nanotechnology. Controlling the self-assembly of nanoscale objects using external stimuli, such as pH, temperature, light, electric potential, or magnetic field, is an important requirement for the preparation of functional and responsive molecular machines for a wide range of potential applications. Here we report cationic multivalent dendrons, with *o*-nitrobenzyl linked spermine surface groups that self-assemble with DNA via multivalent ionic interactions. Cleavage of *o*-nitrobenzyl groups from the dendron framework by optical irradiation results in rapid release of the covalently bound surface groups and non-covalently bound DNA, due to dendron degradation and charge switching multivalency. These results encourage further developments,

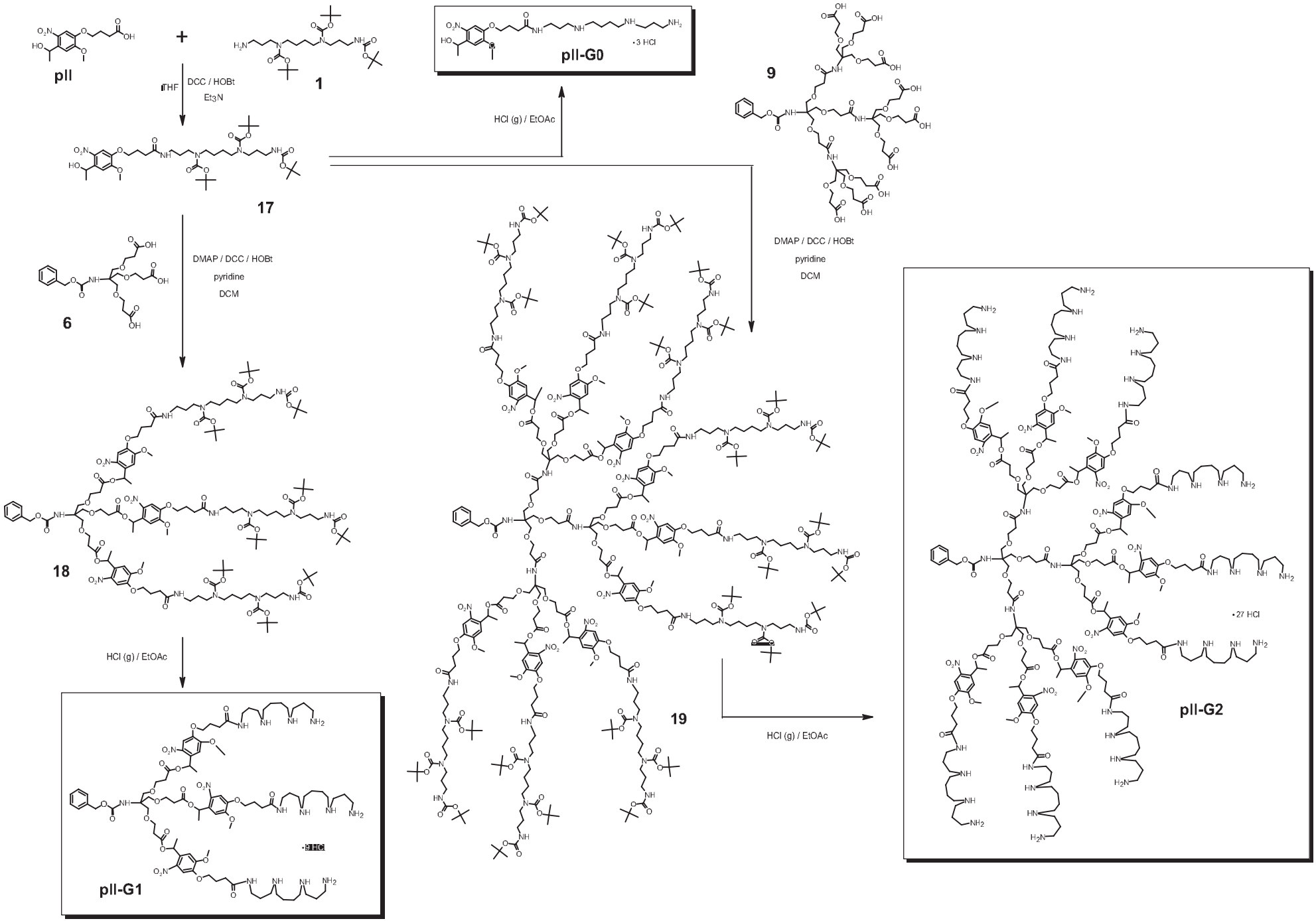
particularly in controlled gene delivery or spatially and temporally controlled DNA storage/release systems, which have been proposed to be of relevance in molecular computing.

IV. Multivalent dendrons that have an *N*-maleimido group at the focal point can be used to construct monodisperse one-to-one protein-dendron conjugates. Here we demonstrate the successful synthesis of series of multivalent protein-dendron conjugates in which the dendron imparts its properties onto the protein to which it is attached: i.e. high-affinity DNA binding (as determined by ethidium bromide fluorescence quenching assay). It is therefore possible to convey DNA affinity to proteins that do not have natural DNA binding ability. Notably, using this approach, **HFBI-G2** is one of the strongest DNA binding proteins ever reported. It is further possible to 'fine-tune' the functionality of the dendron periphery by means of organic synthesis and to further control the interactions between molecules and the self-assembly of nanoscale bioconjugates.

V. Nature has evolved proteins and enzymes to carry out a wide range of sophisticated tasks. Proteins modified with functional polymers possess many desirable physical and chemical properties and have applications in nanobiotechnology. Here we describe multivalent Newkome-type polyamine dendrons that function as synthetic DNA-binding domains, which can be conjugated with proteins. These polyamine dendrons employ naturally occurring spermine surface groups to bind DNA with high-affinity and are attached onto protein surfaces in a site-specific manner to yield well-defined one-to-one protein-polymer conjugates, where the number of dendrons and their attachment site on the protein surface is precisely known. This precise structure is achieved by using *N*-maleimido-cored dendrons that selectively react via 1,4-conjugate addition with a single free thiol group on the protein surface – either Cys-34 of Bovine Serum Albumin (BSA), or a genetically engineered cysteine mutant of Class II hydrophobin (HFBI). This reaction can be conducted in mild aqueous solutions (pH 7.2-7.4) and ambient temperature resulting in BSA and HFBI-dendron conjugates. The protein-dendron conjugates constitute a specific biosynthetic diblock copolymer and bind DNA with high affinity as shown by ethidium bromide displacement assay. Importantly, even the low-molecular-weight first generation polyamine dendron (1 kDa) can bind a large BSA protein (66.4 kDa) to DNA with relatively good affinity. Preliminary gene transfection, cytotoxicity and self-assembly studies establish the relevance of this methodology for *in-vitro* applications, such as gene therapy and surface patterning. These results encourage further developments in protein-dendron block copolymer-like conjugates and will allow the advance of functional biomimetic nanoscale materials.

Synthesis of Target Molecules




pII
1
THF
DCC / HOBt
Et3N
17
6
DMAP / DCC / HOBt
pyridine
DCM
18
HCl (g) / EtOAc
pII-G1
pII-G0
• 3 HCl
HCl (g) / EtOAc
9
DMAP / DCC / HOBt
pyridine
DCM
19
HCl (g) / EtOAc
pII-G2
• 27 HCl

ISBN 978-951-22-9371-1
ISBN 978-951-22-9372-8 (PDF)
ISSN 1795-2239
ISSN 1795-4584 (PDF)