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Biomedical Applications of Peptide-, Glyco- and Glycopeptide Dendrimers, and Analogous Dendrimeric Structures

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Preface

The purpose of this book is to show the progress in the field of synthesis and biomedical applications of peptide-, glyco-, glycopeptide dendrimers and analogous dendrimeric structures. This booklet is intended to graduate students, advanced undergraduates, and scientists working in the area of organic chemistry, biochemistry, nanoscience, biology, medicinal chemistry, and biomedical applications.

Dendrimers and dendritic macromolecules were pioneered in the 1980s. They represent a dynamically expanding field spreading from physics, chemistry, and biology to a broad area of biomedical applications. Dendrimers penetrate to till now isolated areas of science and create an interconnected, logical, mutually influencing scientific network, the dendrimer science (dendrimerology).

The reader will be introduced to a very diverse group of methodologies spanning from chemistry, physics, bioengineering, materials science, biomedical applications, and beyond.

Many problems, when seen from the "dendrimeric point of view," could be overcome if people would use the broad scale of dendrimeric properties and applications. The "cluster effect" ("multivalency") plays a key role and serves as a connecting glue between different structures.

During the last years the number of papers about dendrimers and their applications reached about 1,600 annually, i.e., twice so much in comparison with 2001. That means that the information wave is at the top. Due to space limitation, this book could not cover all papers and reviews and we apologize to researchers involved in the field whose works have not been covered herein. More references can be found in our earlier reviews (2005; 2008a,b,c; 2011).

Prague, Czech Republic
September 2012

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Contents

Acronyms

Nomenclature and abbreviations: Standard nomenclature and abbreviations of amino acids and peptides have been used (Jones, JH (2006) J. Pept. Sci. 12:1–12). Nomenclature and abbreviations of carbohydrates are in accord with IUPAC (McNaught, AD (1996) Pure Applied Chem. 68: 1919–2008). Unless stated otherwise, amino acids are of L-configuration and carbohydrates are of D-configuration. Other abbreviations and notations used are given below.

AB5	AB5 hexameric assembly of cholera toxin consisting of a single copy of the enzymatic subunit A and five copies of the receptor subunit B
Abs	Antibodies
ACE	Affinity capillary electrophoresis
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AFM	Atomic force microscopy
AIDS	Acquired immunodeficiency syndrome
AIE	Aggregation-induced emission
APC	Antigen presenting cell
ASGPR	Asialoglycoprotein receptors on the surface of hepatocytes
ATRP	atom transfer radical polymerization
BARAC	Biarylazacyclooctynone
BBB	Blood-brain barrier
bipy	2,2'-bipyridyl
BNNTs	Boron nitride nanotubes
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
BSI	Back-scattering interferometry
CA	Blood clearance agent
CB	Cucurbituril-based
CCIs	Carbohydrate-carbohydrate interactions
CD	Circular dichroism or cyclodextrin

CE	Capillary electrophoresis
CE-ESI-TOF-MS	Capillary electrophoresis electrospray ionization time-of-flight mass spectrometry
CHD	Chitosan dendrimers
CJD	Creutzfeld–Jakob's disease
CNS	Central nervous system
CNTs	Carbon nanotubes
Con A	*Canavalia ensiformis* agglutinin; Concanavalin A
COSS	$R_8Si_8O_{12}$ cube-octameric silsesquioxanes
CSFV	Classical swine fever virus
CZE	Capillary zone electrophoresis
DC	Dendritic cell
DCLs	Dynamic combinatorial libraries
DC-SIGN	Dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DNP	Dinitrophenyl group
Dns	Dansyl
DOTA	1,4,7,10-tetraazacyclododecane tetraacetic acid
DOX	Doxorubicin
DTPA	Diethylenetriaminepentaacetic acid
DTT	Dithiothreitol
ECM	Extracellular matrix proteins
ELISA	Enzyme-linked immunosorbent assay
ELLA	Enzyme-linked lectin assay
EM	Electrophoretic mobility
EPO	Erythropoietin
EPR	Electron paramagnetic resonance
ESI-MS	Electrospray ionization mass spectrometry
FA	Folic acid
FGF-1	Fibroblast growth factor-1
FGFR	Fibroblast growth factor receptor
FimH	Fimbrial lectin
FITC	5-fluorescein isothiocyanate
FMDV	Foot-and-mouth disease virus
FTICR-MS	Fourier transform ion cyclotron resonance mass spectrometry
FTIR	Fourier transform-infra red spectroscopy
glyco-SAMs	Glyco-self-assembled monolayers
GABA	γ-aminobutyric acid
GAS	Group A *Streptococcus*
GN4C	Glycodendrimer consisting of four GlcNAc residues on calix[4]arene core
GNA	*Galanthus nivalis* agglutinin – snowdrop lectin
Gn	Generation number

GNPs	Gold nanoparticles
GOX	Glucose oxidase
GPC	Gel permeation chromatography
GSS	Gerstmann-Straussler-Scheinker's syndrome
HA	Hemagglutinin
HbA_{1c}	Glycated hemoglobin
HBPO	Hydrophobic poly(3-ethyl-3-(hydroxymethyl)oxetane) core
HIA	Hemagglutination inhibition assay
HIV	Human immunodeficiency virus
hMSCs	Human mesenchymal stem cells
HPAMAM	PAMAM-hyperbranched poly(amido amine) dendrimers
HPLC	High performance liquid chromatography
HPMA	Hyperbranched polyamidoamines
HPTLC	High performance thin layer chromatography
HPV	Human papilloma virus
HRPO	Horseradish peroxidase
HSV	*Herpes simplex* virus
HuPrP	Human prion protein
HUVEC	Human umbilical vein endothelial cells
IL	Interleukin
IFN-γ	Interferon-γ
ITAM	Immunoreceptor tyrosine based activation motif
ITC	Isothermal titration calorimetry
KLH	Keyhole limpet hemocyanin
Lac	Lactose
LCST	Lower critical solution temperature
LecA	*Pseudomonas aeruginosa* lectin LecA (PA-IL)
LecB	*Pseudomonas aeruginosa* lectin speific for L-fucose
LILBID MS	Laser induced liquid beam ion desorption mass spectrometry
LnNPs	$NaGdF_4$:Er^{3+},Yb^{3+} upconverting nanoparticles
LRET	Luminescence resonance energy transfer
MAG	Multiple antigenic glycopeptide
MALDI-TOF MS	Matrix-assisted laser desorption-ionization time of flight mass spectrometry
MALLS	Multi angle laser light scattering
MAP	Multiple antigenic peptide
MD	Molecular dynamics simulations
MDO	Mannosylated dendrimer ovalbumin
MMP-2	Matrix metalloproteinase-2
Mϕ	Monocyte-macrophage lineage
MP	Methylprednisolone
MRI	Magnetic resonance imaging
MSC	Marrow-derived mesenchymal stromal cells
MSCs	Bone marrow-derived mesenchymal stem cells
MS/MS	Tandem MS

MT1-MMP	Membrane type 1-matrix metalloproteinase
nAChR	Nicotinic acetylcholine receptor
NAC	*N*-alkyl cysteine
NA	Neuraminidase
NCAM	Neural cell adhesion molecule
NMR	Nuclear magnetic resonance
NPs	Nanoparticles
NT	Neurotensin
nvCJD	New variant of Creutzfeld-Jakob's disease
OBOC library	One-bead-one-compound library
ODNs CpG	Synthetic oligodeoxynucleotides (ODNs) with the CpG-motifs
OSA	Oncogenic suppression activity protein
OVA	Ovalbumin
$P2Y_{14}$	G protein-coupled receptor activated by uridine-5'-diphosphoglucose
paCDs	Polycationic amphiphilic cyclodextrins
PAGE	Polyacrylamide gel electrophoresis
PA-IL	*Pseudomonas aeruginosa* lectin I
PA-IIL	*Pseudomonas aeruginosa* lectin II
PAMAM	Polyamidoamine dendrimer
PAMAM-SAH	PAMAM-succinamic acid dendrimers
PANINT's	Polyaniline nanotubes
PDEAEMA	Poly(2-(*N,N*-diethylamino)ethyl methacrylate)
pDNA	Plasmid DNA
PD	Parkinson's disease
PDT	Photodynamic therapy
PEC	Pentaerythritol core
PEG	Polyethylene glycol
PePOs	Pentaerythritol phosphodiester oligomers
PGLD	Bioactive polyglycerol dendrimers
PLA	Poly(L-lactide)
PLGA	Poly-D,L-lactide-co-glycolide
PNA	Peanut agglutinin, a galactose-binding lectin from *Arachis hypogaea*
PPI	Poly(propylene imine) dendrimer
proMMP-2	Promatrix metalloprotease-2
PrP^{C}	Cellular prion protein
PrP^{Sc}	Scrapie isoform of prion protein
PSMA	Prostate-specific membrane antigen
PSP	Pseudostationary phase
PTA	*Psophocarpus tetragonolobus* agglutinin
QqTOF MS	Quadrupole-quadrupole time-of-flight mass spectrometry
RAFT	Regioselectively addressable functional template
Rhod	Rhodamine
RIP	Relative inhibitory potency

RP-HPLC	Reverse-phase HPLC
RVG	Rabies virus glycoprotein
SALs	Sugar-assisted ligations
SAMs	Self-assembled monolayers
SAv	Streptavidin
SAXS	Small-angle X-ray scattering
scFv	Single chain fusion of light and heavy chains in variable region of mAb
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
SDS	Sodium dodecylsulfate
SEC	Size exclusion chromatography
siRNA	Small interfering RNA
SLNs	Solid lipid nanoparticles
SOCs	Sequential oligopeptide carriers
SPR	Surface plasmon resonance
SRCD	Synchrotron radiation-based circular dichroism
STM	Scanning tunneling microscopy
SWNTs	Single-walled carbon nanotubes
Syk	Spleen tyrosine kinase
TACA	Tumor-associated carbohydrate antigen
TAT	The transactivator of transcription of human immunodeficiency virus
TEM	Transmission electron microscopy
Th1	Distinct type of T-helper cell
TK	Thymidine kinase
TLR2	Toll-like receptor 2
TNF-α	Tumor necrosis factor α
TPEs	Mannose substituted tetraphenylethenes
TRIS	Tris(hydroxymethyl)aminomethane
UDPGA	Uridine-5'-diphosphoglucuronic acid
UPLC	Ultra performance liquid chromatography
WGA	Plant lectin, wheat germ agglutinin

Chapter 1
Introduction

Dendrimer-like structures exist in the universe and on our planet Earth hundreds of million years. They are present in living and nonliving things, e.g., nerves, nervures, veins, gekon's pads, treetops, roots of a tree, the supernova remnant Crab Nebula, forked lightning, river basins, and snow crystals [8]. Their size is from light-years to kilometers, to microscale and nanoscales. On the basis of thermodynamic data, it was shown that branched structures are more stable than the linear ones. For example, 2,2-dimethylpropane is much more stable than linear *n*-pentane [10]. Branching is a general tendency in nature. Therefore, when something can get branched, it will get branched. The branched structures, including dendrimers, have properties that could result in faster and more efficient transfer, dissipation, and distribution of matter and/or energy.

The word dendrimer is derived from the Greek word dendron ($\delta\acute{\epsilon}\nu\tau\varrho o$) meaning "tree" or "branch," and meros ($\mu\varepsilon\varrho o\varsigma$) meaning part [1, 7]. The definitions of dendrimer differ depending on the specialization of authors. Some of the definitions are as follows:

Dendrimers are versatile, derivatizable, well-defined highly branched three-dimensional macromolecules (chemical polymers) with characteristic compartmentalized structures, the sizes and physicochemical properties of which resemble those of biomolecules, e.g., proteins, nucleic acids, and polysaccharides, and a tendency to adopt a globular shape once a certain size is reached. Dendrimers have highly controlled structures, a large number of controllable "peripheral" functionalities, and a single molecular weight and can be characterized as chemical entities (with some limitations). They contain different cores, branches, and surface groups and have different number of generations [1, 5, 12, 13]. For more definitions and reviews see [11, 21]. The composition, valency, generation, structure, etc. determine their physicochemical properties and biological activities.

Dendrimers are monodisperse polymers that adopt a globular three-dimensional shape as the generation number (Gn) increases. These branched macromolecules have a well-defined core, interior region layers connected by branches, and an exterior crimpy surface. This affords a high surface area-to-volume ratio. The

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number of end groups multiplies with each successive generation. The properties of the dendrimer are therefore more strongly influenced by the nature of the end groups. The structural and chemical attributes of dendrimers create unique physical and chemical properties [9].

Schluter and Rabe [20] used more lyrical approach. They describe dendrimers as "a jungle of entangled branches traversed by winding trails which lead to sweet fruits and bright blossoms." Wandering around these trails, the thicket's interior is reached as well as a way back is found. This thicket plays a role of regularly branched and densely packed structures, whereas the role of trails is empty space filled by solvent. The fruit and blossoms stand for synthetically, photochemically, or electrochemically addressable species. Furthermore, they can play a role of catalytically active sites. Transport processes are depicted in this model as a motion to and from the trails.

Up to 1984 there were only three polymer architectures: linear, cross-linked, and branched-type configurations. Dendrimers create a new "dendritic architectural state" which is a new, fourth class of polymer architecture. Dendritic polymers can be classified into four dendritic subgroups: (1) random hyperbranched polymers, (2) dendrigraft polymers, (3) dendrons, and (4) dendrimers [22, 24]. These four subgroups determine the rate of relative structural control of these dendritic architectures including their increasing symmetry. The majority of peptide dendrimers described in the literature is based on the multiple antigenic peptides (MAP) that contained only two of the three structural features: branching units and surface functional groups. Interestingly, MAPs contain both α- and ϵ-peptides. Furthermore, MAPs are often synthesized without a core. Thus, more appropriately the term "dendrons" should be used rather than dendrimers. Nevertheless, for convenience in this book, we call all branched polypeptide constructs as dendrimers [19]. Especially, high-generation dendrons adopt structures resembling dendrimers, with only local inconsistency in missing core area.

The amount of dendrimeric structures is unlimited and in this book therefore only peptide-, glyco-, and glycopeptide dendrimers will be mentioned, including their biomedical applications. Figure 1.1 shows fast increase of publication activity during the last decade. The topic of glycopeptide dendrimers shares approximately 10–20% of the set of dendrimers depending on the year; the most recent shares are close to 20%.

The sets of dendrimers and hyperbranched polymers partly overlap [8, 23]. They differ in regularity of structure, synthetic availability, and chemical variability. Dendrimers are more regular and better defined but more expensive (= more difficult to prepare). This book covers mainly the topic of dendrimers with highlights of more important discoveries in the field of hyperbranched polymers.

Dendritic structures represent a new class of polymers, first reported by Vogtle et al. They were named cascade molecules [2]. This field developed to larger dendritic structures and this class of compounds was renamed as dendrimers [1, 6, 25, 26]. For more details see [8, 11, 21].

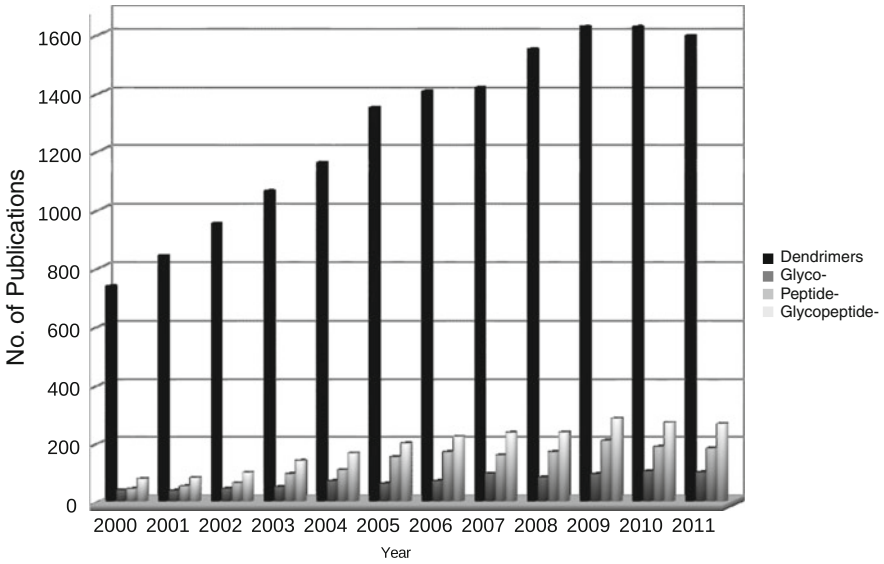

Fig. 1.1 Trends in publications of dendrimers, and various subsets such as glyco-, peptide-, and glycopeptide-dendrimers. Data were obtained from the Web of Science database in Jun/20/2012

Scope and Limitations

Like in [14] we use the term "dendrimer" in a more free sense, i.e., as a more or less branched, chemically defined, and characterized structure, the biological, chemical, and physical properties of which not only are the sum of the given substructures but create a new quality. By other words, the unifying ideas in dendrimer chemistry and biology are the "cluster effect" or "multivalency," together with the branched structure [3, 4, 11, 21]. The choice of how to organize this book was difficult. As chemists, we decided for classification based on dendrimer physicochemical properties, synthetic pathways (convergent, divergent), and their structure. As a consequence, crucial compounds and their activities could be omitted. To prevent these losses of key topics, the second part of this book contains applications and biomedical properties of dendrimers. Since the field of dendrimers contains more than 1600 new items per year (Fig. 1.1), only some selected examples are given without demand for completeness. In some cases one item is located at more places, e.g., bacteria, lectins, and drug delivery. The term MAGs will be used for branched compounds with sugar on the surface in analogy with MAPs [15, 19]. Other types of compounds will be named as glycopeptide dendrimers.

The field of glycopeptide dendrimers was opened in 1993 by Roy [16–18]. The sugar attached to the dendrimer surface was sialic acid that was introduced to confer strong inhibitory properties against flu virus hemagglutinin, which is a lectin-like protein recognizing α-sialosides on respiratory mucins.

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Part I
Chemistry and Structure of Dendrimers

Dendrimers represent a class of polymeric architecture with very regular shape. Their size corresponds to dimensions of biologically important molecules such as enzymes. They are capable to form supramolecular structures, self-healable hydrogels, and outstanding catalysts. Hence, their properties have a wide application in material and biological sciences.

The first part of this book describes basic nomenclature terms and many approaches on how to tackle the challenge of syntheses and characterizations of perfectly regular molecules (dendrimers). This includes ligation, click, and Lego chemistries. The basic classification of dendrimers is also provided.

Moreover, the use of dendrimers in catalysis is also emphasized. Their unique properties are driven by the structure and by the difference in polarity among solvent, substrate, product, dendrimeric shell, and dendrimeric core (interior). Thus, diffusion can push hydrophobic substrates close to hydrophobic core in water, whereas hydrophilic shell of dendrimers serves for solubilization of the catalysts. Hydrophilic product is then excreted from active site to bulk water. Using these principles, one can construct artificial enzymes.

Chapter 2
Definition of Terms and Nomenclature

Basic terms and nomenclature of dendrimers have been covered by many excellent reviews [6, 11, 12, 20, 21, 37, 39, 86, 96, 97, 101, 110]. Detailed nomenclature rules for dendritic molecules ("cascadane-nomenclature") based on the principles of dendrimer terms [73] have been elaborated [33]. Without any doubts, it is not easy to create a nomenclature for dendrimers, like a nomenclature for carbohydrates, and peptides. The topic of dendrimer chemistry is fantastically diverse; therefore, exact classification is very difficult or even impossible. When possible, then the name is complicated, long, or difficult to understand, especially for immunologists. Besides, MAP (multiple antigenic peptide) and MAG (multiple antigenic glycopeptide) dendrimers use their own nomenclature. The figure with chemical formula is still the best information. Other possibility is to use a combination of well-known trivial names like glucose, and lysine. Independently on the above facts, the authors [33] deserve admiration.

A hexadecavalent MAP core can be described simply and clearly as R_{16}-Lys_8-Lys_4-Lys_2-Lys-OH, where R = peptide antigen. The same MAP with R = H can be represented as {[H-Lys-Lys(&1)-Lys(&2)-Lys(&3)-OH][H-Lys-Lys(&4)-Lys(&5)&3][H-Lys-Lys(&6)&2][H-Lys-Lys(&7)&5][H-Lys&1][H-Lys&4][-H-Lys&6][H-Lys&7]} [103].

For example, the PAMAM dendrimer can be depicted as linear representation (Fig. 2.1). Another way of PAMAM nomenclature was proposed [23].

Another approach is fractal notation [64], a concise descriptive system for symmetrical dendrimers and other, simple symmetrical molecules. Fractal notation was applied also for nearly symmetrical dendrimers [65]. Fractal notation derives its qualities by taking advantage of the symmetry of dendritic molecules. The integrity of the system can be preserved also in nearly symmetrical dendrimers, which can be described as perturbations of the symmetrical parent molecules (Fig. 2.2).

Tomalia [110] proposed a systematic framework in order to unify and define nanoscience on the base of historic first principles and step logic that led to a "central paradigm" (i.e., unifying framework) for chemistry of traditional elemental/small molecules. The proposed "nanomaterials classification road map" divides

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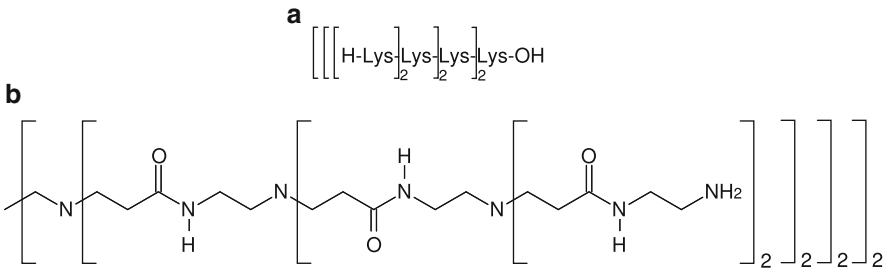

Fig. 2.1 Linear representation of classical MAP (**a**) and G3 PAMAM dendrimer (**b**). The extended structures are depicted in Fig. 2.2

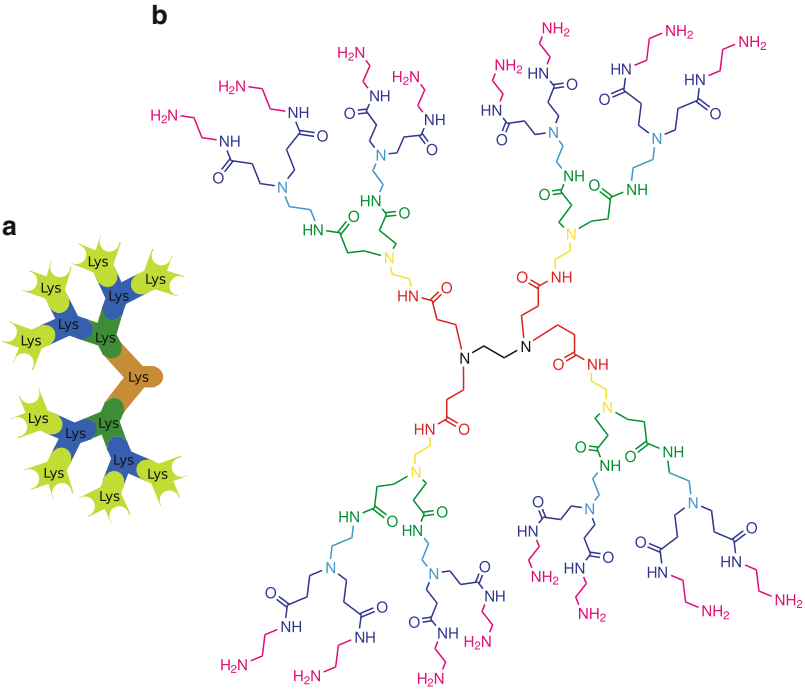

Fig. 2.2 Example of generation definition for G3-MAP (**a**) [92, 105] and G3-PAMAM (**b**) [10]. In the case of MAP (dendron), every generation is defined with color code starting from *brown* Lys (G0) leading through *dark green* (G1), *blue* (G2), and *light green* (G3) Lys residues. For PAMAM case (dendrimer), even halves of generations are depicted. *Black core* (G0) is surrounded by *red branches* (G0.5) which continue to *yellow ones* (G1). Next half generation is *green* (G1.5) and whole generation is *cyan* (G2). Finally, *dark blue* and *magenta* colors stand for G2.5 and G3, respectively

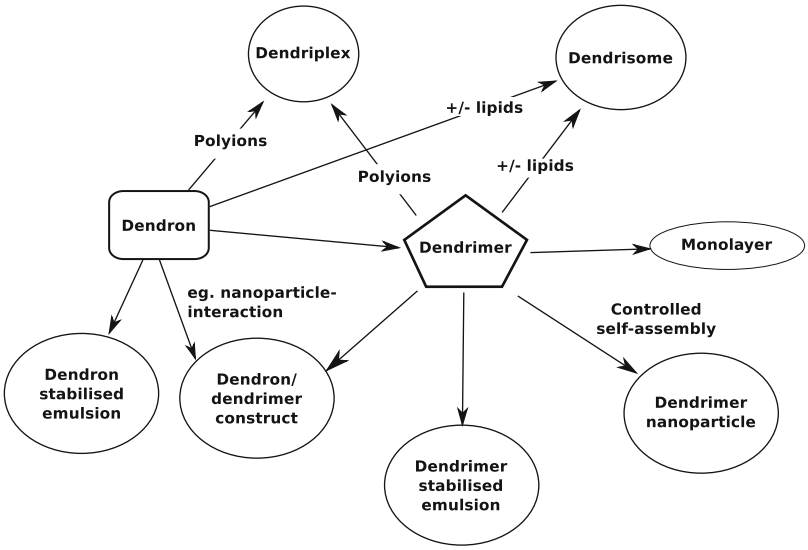

Fig. 2.3 A schematic depiction of the variety of relationships between the primary dendron and dendrimer structures and their aggregated states. Adapted and extended from [1, 75]

all nanomatter (including dendrimers) into Category I: discrete, well-defined and Category II: statistical, undefined nanoparticles. The proposed nanoperiodic table(s) is a milestone, like the Mendeleev table of elements, and can be used for predicting important risk/benefit borders in the nanoscience and dendrimer-based field. This predictive ability represents a breakthrough in theoretic approach to dendrimers.

For other nomenclature approaches in the area of nanoparticle and dendrimer classification see [50, 69, 85, 93, 103, 107]. Till now, there is not one simple, unambiguous, generally applicable, and worldwide acceptable dendrimer nomenclature, like IUPAC-IUB rules, which must be respected in every journal.

2.1 Dendriplexes and Dendrisomes

DNA can form nanostructures with cationic dendrons and dendrimers. These complexes have been termed dendriplexes [1, 82, 84]. However, similar structures can be probably formed from anionic dendrimers and linear polycations. Schematic interrelationships between dendrons, dendrimers, and their aggregated states (monolayers, dendrisomes, dendriplexes, nanoparticles, etc.) are shown in Fig. 2.3.

Small vesicular aggregates that are formed in water from cationic lipidic lysine MAP dendron with the appropriate hydrophile–lipophile balance were termed dendrisomes. Dendrisomes are resembling cationic liposomes and are able to

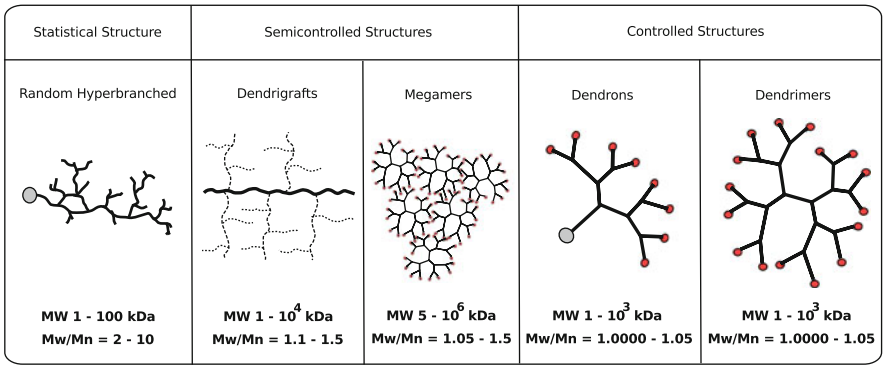

Fig. 2.4 Schematic representation of random hyperbranched polymers, dendrigrafts, megamers, dendrons, and dendrimers. Adapted from [7, 53, 54, 75, 104, 108–111, 114]

encapsulate water-soluble, negatively charged compounds [1]. Both dendriplexes and dendrisomes are used as drug delivery systems and vectors [67].

2.2 Dendrigraft

For dendrimer synthesis, traditional monomers are generally employed. Dendrigrafts are prepared from reactive oligomers or polymers. Therefore, dendrigrafts are generally much larger (Fig. 2.4) than dendrimers [25, 31, 108, 109, 111–113]. Some authors use the term "dendronized polymer" [51, 58].

2.3 Glycotope

The term "epitope" is used often in immunology, peptide, and protein chemistry. Epitopes are sites on an antigen that interact with specific antibodies. They can be either conformational or sequential. In the area of glycopeptides, glycodendrimers, MAGs, and glycobiology, the term "glycotope" is used. Glycotope is a three-dimensional carbohydrate (or glycopeptide) epitope in which carbohydrates play a decisive role in basic immunological recognition processes (antibodies, self–nonself, interaction with T and B cells, etc.). Its activity is governed not only by the carbohydrate part but depends also on the peptide or protein backbone and also on the amino acid, to which the glycotope is bound (Ser, Thr) [27, 56, 59, 81, 88, 97, 99, 120, 121, 127–130, 133]. Some respected authors [15, 29, 41, 43, 58, 77] do not use the term anyway. Many terms, e.g., glycotope, sugar epitope, carbohydrate epitope, glycopeptide epitope, polyvalent glycotope, glycocluster, and glycodomain more or less coincide. We will use the term glycotope.

2.4 Glycocluster

Sterical arrangement of two or more glycotopes, which can be in the form of dendron or dendrimer, is called glycocluster. The clustering leads to the amplification of the given biological or physicochemical activity. The amplification factor (activity increase overcalculated to one active unit, e.g., Glc) is a few orders of magnitude higher in comparison with the sum of the individual contributions. For more details see Sect. 2.6; [2–5, 14, 16, 18–20, 22, 24, 36, 38, 40, 42, 44, 45, 49, 52, 55, 58, 60, 61, 66, 71, 72, 75, 79, 80, 87–90, 94, 95, 98, 100, 102, 115, 119, 124, 125, 132, 134].

2.5 Glycocluster–Cluster

Complex glycoclusters are sometimes called as "glycocluster–cluster" [95]. The transformation of a simple sugar unit to a glycocluster unit in order to synthesize a novel "glycocluster–cluster" is accomplished using a glycocluster–cluster unit (cluster) in which a condensed glycocluster unit is connected with a cluster chain unit. By other words, e.g., dendrimers with valences 3,4, etc. are grouped in 3,4,5, etc. copies to a common core, shell, or backbone. On the other hand, other authors do not use designation "glycocluster–cluster" [20]. They prefer using the term glycoclusters also for complex molecules, e.g., with six branches having three carbohydrates on each branch, bearing together 18 α-mannopyranoside residues.

2.6 Cluster Effect and Multivalency

In general, interactions between saccharides and peptides or proteins are weak. Isolated carbohydrate–protein interactions are typically very weak with K_D values in the range of 10^{-3}–10^{-6} M. The nature compensates for the weakness of these isolated interactions by tending to cluster together multiple copies of carbohydrate ligands and their receptors [30, 97] (Fig. 19.1).

The activity of a tested compound (glycodendrimer, glycocluster, peptide dendrimer, etc.) can be generally expressed in three ways [18]: (1) Minimum concentration [e.g., in μM] required to inhibit the studied activity (e.g., agglutination of erythrocytes). (2) Relative potency, which is calculated as the ratio of the monovalent reference value to the ligands value. (3) Relative potency per carbohydrate (peptide), calculated as the ratio of the relative potency to the dendrimer (cluster) valency. By other words relative potency = IC_{50} (monosaccharide)/IC_{50} (inhibitor) and relative potency/sugar = relative potency/n [13]. The third way, relative potency per 1 carbohydrate (peptide), seems to be the most exact and explanative [18].

A bacterial lectin (PA-IL) from the opportunistic pathogen *Pseudomonas aeruginosa*, which is involved in recognition processes of glycoconjugates on human

tissues, was studied [18]. The dodecavalent fullerene glycoclusters obtained were tested as ligands of PA-IL and for their competing potential with its binding to glycosylated surfaces. The affinities tested by hemagglutination inhibition assay (HIA), surface plasmon resonance (SPR), and enzyme-linked lectin assay (ELLA) displayed a significant "glycoside cluster effect" with up to a 12,800-fold increase in binding (relative potency compared to Gal) and nearly 1100-fold increase of relative potency per carbohydrate. Both values are classical example of cluster effect, i.e., with growing valency of the cluster (dendrimer) the activity of the multivalent compound grows exponentially. This has some limits, given by the sterical demands of the surface groups on the dendrimer and also by the shape and dimensions of the counterpart (lectin, etc.).

Another convincing example for a cluster effect in FimH binding was shown by employing mannosylated lysine-based MAGs [70]. The relative inhibitory potencies of the di-, tetra-, octa-, and hexadecavalent mannosylated MAGs increased with growing generations from 455 over 2000 and 3571 to 11111, respectively [44]. In spite of the fact that the avidity did not grow logarithmically, it rose to a much greater extent than it would by a linear increase with respect to the increasing valency. This increase of activity is called "cluster effect" or "multivalent effect" [10, 20, 34, 60, 63, 75, 87, 90, 91, 97, 116, 117, 126, 134].

Roy's team [91] prepared sialosyl MAGs with valencies of 2, 4, 8, and 16. Sialic acid was bound to the lysine branches by SCH_2-CO-Gly-Gly spacer. Binding properties of these MAGs with the plant lectin wheat germ agglutinin (WGA) were studied in a direct ELLA using horseradish peroxidase (HRPO)-labeled WGA . The octa- and hexadecavalent MAG had best binding properties. In an inhibition test using sialylated MAGs as coating antigen and HRPOWGA, all MAGs performed excellent inhibitory capacities (10^6 times better than a monosialoside). The most powerful inhibitor was the hexadecavalent MAG.

Dramatical enhancement of dissociation constant between folate-binding protein and folate-G5 PAMAM dendrimer was described [47]. The binding avidity was improved by cluster effect up to 170,000 times.

The terms "cluster glycoside effect" [60] or "glycoside cluster effect" [18, 48], multivalent effect [46], multivalent glycotope [99], and clustering effect [9] are used only seldom and we will therefore use the most common term cluster effect [8, 18, 32, 44, 46].

We have not found exactly defined quantitative limits above which the activity increase can be called "cluster effect." For more information about cluster effect and multivalency see [1, 6, 17, 20, 26, 35, 39, 46, 48, 57, 68, 74, 75, 83, 96, 97, 99, 101, 106, 118, 122, 131].

2.7 Macromolecular Effect

Multivalency or cluster effect is not able to explain all interactions. To solve this problem, Lindhorst and her team proposed that comprehension of fimbriae-mediated bacterial adhesion might require at least two different approaches [28]. One attitude

deals with results obtained from ELISA or hemagglutination inhibition assays. The observed inhibition of bacterial adhesion could be neither rationalized on the basis of the known crystal structure of FimH nor interpreted in the sense of a classical "cluster effect." Instead, the inhibition of bacterial adhesion to the glycocalyx or a glycocalyx mimetic should be more likely explained by a "macromolecular effect" [28]. The inhibitory potencies can be correlated to features which are typical for macromolecules and the interactions they form, rather than for distinct molecular epitopes [28]. The macromolecular effect was discussed also in connection with fucosylated pentaerythrityl phosphodiester oligomers (PePOs) and their binding to *Pseudomonas aeruginosa* lectin (PA-IIL) [66]. Positive macromolecular effect during enhanced gelation of a dendritic gelator was described [135]. The macromolecular effect during NMR analysis of Gd(III)-based contrast agent influenced T1 relaxivity [62].

2.8 Sugar Ball

"Sugar balls" provide a spherical platform for a globular multivalent presentation of ligands with many carbohydrate residues in the peripheral region of the dendrimer [76, 78, 123]. It is based on fullerenes, quantum dots, or other spherical nanoparticles, wrapped to sugar shell.

2.9 "Smart" Glycodendrimers

Glyco- and glycopeptide dendrimers containing labile functionalities which can easily interconvert in solutions (disulfides, imines, hydrazones, metal coordination, etc.) are left to equilibrate in order to select one another to best fit to the binding site of interest [87]. This strategy was called "dynamic combinatorial chemistry" and was used in the preparation of dynamic combinatorial libraries (see Chap. 8).

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Chapter 3
Sugar Code (Glycocode)

The vast majority of signal transduction processes in most living organisms are caused by four sorts of biomolecules: nucleic acids, proteins, glycoconjugates, and lipids. The sequence and structure of nucleic acids as well as peptides and proteins have been extensively studied, including different sorts of interactions and functions (Fig. 3.1) [27, 31]. Genomics [16, 19, 25, 28], proteomics [15, 24, 29, 35, 38, 39], glycomics [4, 8, 12, 17, 22, 26, 27, 31–34, 37], and lipomics [7, 9, 18, 21, 36] represent four logically, chemically, and biologically interconnected areas of research approaches to living organisms. The development in glycomics, in comparison with genomics and proteomics, was more demanding owing to the monumental growth of possible isomers and structural variations. This "heterogeneity" (variability) stems largely from their inherent features of biosynthesis. The term glycomics was introduced to describe glycobiology and the interaction of carbohydrates with the other two major classes of biopolymers.

In analogy with the term "proteome," the term "glycome" has been coined for the glycan repertoire of an organism. Also, in the wake of "genomics" and "proteomics," the word "glycomics" has become the trendy term for the characterization by function and structure of glycans in the studied system [8, 12, 22].

Phosphodiester and peptide bonds create in general linear structures, which are completely defined by their sequences. Its permutations are therefore the only source of coding capacity of nucleic acids and peptides, respectively. The situation for carbohydrates is absolutely different [10, 11, 13]. There are four additional parameters in glycans which extremely increase their coding capacity: (1) positions of linkage points (e.g., 1→2, 1→3, 1→4, or 1→6); (2) anomeric configuration (α or β; glycogen/starch differ from cellulose only in this parameter); (3) ring size (pyranose or furanose); and (4) branching of oligomers larger than trimers [10, 13]. For complete characterization of the structure of any saccharide, besides the sequence, all the parameters listed above (first and second dimensions of the sugar code) have to be defined. Therefore, α-lactose is not simply galactosyl-glucose (Gal-Glc), but β-Gal*p*-(1→4)-α-Glc*p*. Structural determination of saccharides is a few orders of magnitude more demanding and time consuming than for corresponding

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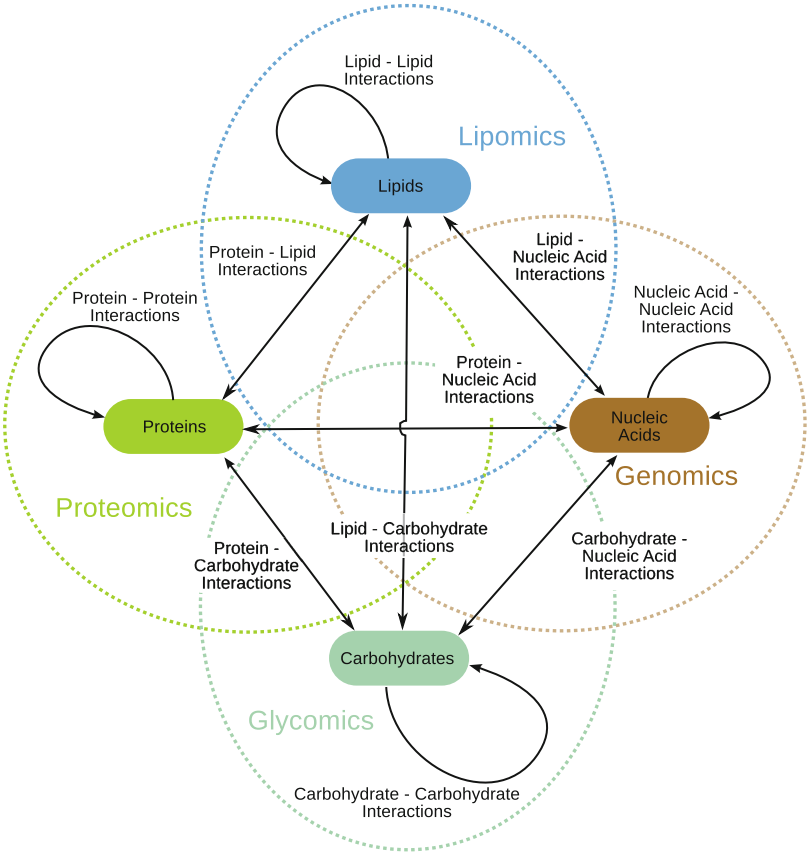

Fig. 3.1 Different types of interactions between the four main biomolecules, extended from [23]

nucleotides or peptides. Owing to these facts, breaking of the sugar code was delayed behind the deciphering of the other two coding systems [10, 11]. The diversity generated by these factors [10, 11, 13] is responsible that carbohydrates are unbeatable in information potential, compared with proteins and nucleic acids.

The ability to store information in nucleic acids, proteins, and carbohydrates was calculated [10–13, 20, 23]. For trimers of nucleotides with the four pyrimidine and purine bases there are 64 sequence permutations (4^3). Peptides score better. The trimers of the standard 20 amino acids can form 8,000 isomers (20^3). Under the same conditions, sugars possess 9,000,000 isomers. In the case of oligomers larger than trimers, branching of saccharides must be reflected in the calculation. The number of hexamers for peptides is 64,000,000 (20^6) and looks much better in comparison with 4,096 (4^6) hexanucleotides, but the number of isomeric hexasaccharides including branching is 1.44×10^{15}. Therefore, carbohydrates are the best high-density coding system. This sort of coding (language) has been named glycocode

resp. sugar code [1, 5, 14, 32]. It represents the complex information pool that carbohydrate structures are able to express. Monosaccharides as building blocks for oligo- and polysaccharide synthesis represent therefore high-capacity information-storing and coding units, creating the third alphabet of life. The amount of information carried by glycopeptide dendrimers or glycodendrimers, in comparison with peptide dendrimers, is therefore much higher in all parameters, including structural variability, complexity, spectrum of biological activities, etc.

Some authors use the term glycocode [1, 23], but the term sugar code [2, 10, 11, 13, 14] is used more often.

Lectins represent one of the best tools for the molecular understanding of the sugar code [3, 14].

The topics of sugar code, glycodendrimers, and different sorts of nanoparticles partly overlap. Especially, carbohydrate-mediated molecular recognitions using nano-vehicles have a deep impact on medicine and open a new area of biomedical applications both *in vitro* and *in vivo* [6, 30].

For a fast orientation and context see Fig. 2.3.

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Chapter 4
Classes of Peptide-, Glyco-, and Glycopeptide Dendrimers

It is a difficult task to create a simple, universal, clear, unambiguous, logical, and generally applicable classification or even nomenclature of different sorts of dendrimers. In general, dendrimers contain a core, branches, and surface functional groups. Sometimes the core is missing (e.g., MAPs), or the branches are missing (RAFTs). The classification can stress either the shape of the core, branches, or surface groups (linear, cyclic) or the chemical nature of the core (fullerene, porphyrin, calixarene, cyclodextrin, RAFT, etc.) or branches (peptide, carbohydrate, PEG, PAMAM, etc.)

Another way of classification is in accord with globular structure, cluster effect, and polyvalency. Our classification is limited mainly to peptide-, glyco-, and glycopeptide dendrimers.

It is necessary to explain three partly overlapping terms: core, scaffold, and cluster. Nearly all molecules exhibiting at least two copies of a reactive group can theoretically serve as core or scaffold for the synthesis of different sorts of dendrimers [9, 25, 39, 53, 95, 117, 118, 140, 156, 178, 191, 201]. The term cluster is used mainly for the whole branched dendrimer molecule [4, 9, 25, 39, 95, 117, 151, 156, 178, 195] and only exceptionally in the sense of a core.

We tried to provide some classification of dendrimers; however, the wideness of the field is beyond the scope of this book. Our classification of selected dendritic types is shown in Table 4.1 and is based on core type.

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Table 4.1 Classes of dendrimers

Class	Subclass	Subsubclass	Type	Reference
Linear	Acyclic (brush)	×	Linear polycarbonates	[170]
×	×	×	Cellulose dendrimers	[133]
×	×	×	Chitosan dendrimers	[147, 196, 202, 209]
×	×	×	SOCs	[3, 78, 164, 192, 207]
×	×	×	poly-L-Lys	[96]
×	×	×	TACA brush	[36, 48, 71, 130, 197–199]
×	×	×	Other brush dendrimers	[14, 29, 30, 55, 100, 111, 126, 150, 157, 168, 171, 174, 185]
×	Macrocyclic (necklace)	×	RAFT	[5, 25, 39, 40, 95, 191]
×	×	×	Cyclodextrin	[9, 25, 34, 87, 95, 117, 125, 138, 180, 196, 204]
×	×	×	Calix[4]arenes	[15, 22, 23, 25, 39, 52, 72, 95, 117, 125, 138, 163]
×	×	×	Resorcarene	[125, 166]
×	×	×	Porphyrin	[9, 22, 25, 33, 35, 39, 98, 101, 107, 117, 141, 189]
×	×	×	cyclam (1,4,8,11-tetraazacyclotetradecane) based dendrimers	[9, 25, 169]
Starburst	acyclic	×	PPI dendrimers	[7, 9, 16, 19, 21, 25, 35, 60, 95, 116, 118, 123, 132, 135, 160, 161, 190, 201]
×	×	×	PAMAM	[8–11, 21, 25, 27, 35, 44, 72–74, 76, 82, 94, 95, 109, 110, 118, 123, 136, 148, 152, 153, 158, 160, 172, 181, 182, 201, 205, 206]
×	×	×	MAPs	[1, 9, 17, 35, 38, 41, 58, 70, 75, 91, 102, 103, 105, 112, 115, 119, 120, 140, 143, 155, 156, 158, 175, 176, 183, 186, 187, 193, 203]

×	×	×	MAGs	[6, 9, 12, 25, 45, 46, 61, 62, 79, 81, 104, 128, 156, 178]
×	×	×	Carbosilane-based dendrimers	[21, 25, 35, 64, 95, 117, 122, 124]
×	×	×	Tris(2-aminoethyl)amine	[56, 61]
×	×	×	2,2-bis(hydroxymethyl)-propionic acid	[149]
×	×	×	Glycerol	[4, 20, 62, 90, 93, 95, 140]
×	×	×	Hyperbranched polyglycerol	[129, 147, 201]
×	×	×	*N*-{tris[(2-carboxyethoxy)methyl]} methylamine	[85]
×	×	×	Tetra(hydroxymethyl)-methane	[30, 62]
×	×	×	Tris(hydroxymethyl)C−NH_2	[25, 194]
×	×	×	Tris(carboxyethyl)C−NH_2	[67]
×	×	×	Tris(2-aminoethyl)C−NH_2	[89]
×	×	×	Tetra(aminomethyl)methane	[25]
×	×	×	Tri(aminoethyl)amine	[25]
×	×	×	Pentaerythritol	[2, 4, 25, 35, 49, 62, 66, 95, 113, 117, 156, 184]
×	×	×	Sorbitol	[179]
×	Cyclic	Aromatic cores	Benzene	[25, 35, 54, 92, 114, 165]
×	×	×	Thiophene	[154]
×	×	×	Binaphtyl	[137]
×	×	×	Anthracene	[144]

(continued)

Table 4.1 continued

Class	Subclass	Subsubclass	Type	Reference
×	×	×	Hexaphenylbenzene	[25, 26]
×	×	×	Squarate	[25, 62, 95, 167]
×	×	×	Cyclotriphosphazene dendrimers	[25, 35, 50, 80]
×	×	Alicyclic cores	Galactose (octopus)	[134]
×	×	×	Mannose (octopus)	[62, 134]
×	×	×	Glucose (octopus)	[25, 62, 69, 95, 138, 179]
×	×	×	Fructose	[63]
×	×	×	Inositol	[25, 95, 97]
×	×	×	Oligosaccharide cores (octopus) with raffinose	[42, 95]
×	×	×	Hyperbranched 5,6-glucan	[177]
×	×	×	Adamantane	[117]
×	Hybrid	Aromatic cores	Tetraphenylethene	[146]
Body	Sphere or ellipsoids	×	Fullerene	[9, 24, 25, 39, 99, 106, 117, 121, 125, 145, 173]
×	Cube	×	$R_8Si_8O_{12}$ cube-octameric silsesquioxanes (COSS)	[25, 68]
×	Ring	×	Cyclophane	[65]
×	×	×	Cucurbituril-based (CB) carbohydrate clusters (carbohydrate wheels)	[25, 88, 95]
×	Tube	×	Single-walled carbon nanotubes	[25, 31, 33, 43, 57, 189, 200, 208]
×	×	×	Single-walled boron nitride nanotubes	[32]

Supramolecular	Matalic complexes	×	×	[9, 39, 135]
×	×	×	Tris(2,2-bipyridyl)Ru(II)	[9, 83, 84, 86]
×	×	×	Tris(2,2-bipyridyl)Ru(III)	[25]
×	×	×	Tris(2,2-bipyridyl)Fe(II)	[25]
×	×	×	Ferrocene	[117, 201]
×	Metallic nanoparticles	×	Pt/Pd nanoparticles	[54]
×	×	×	Gold nanoparticles	[18, 37, 39, 43, 51, 59, 108, 127, 132]
×	Metal-oxide nanoparticles	×	Aluminum oxide	[131]
×	×	×	Iron oxides (Fe_3O_4 and γ-Fe_2O_3)	[188]
×	Rotaxanes (string of pearls)	×	Cyclodextrins on linear polymer	[13, 34]
×	Self-organized multicomponent systems	Soft nanoparticles	×	[142]
×	×	×	Dendriplexes	[77, 139, 159, 161, 162]
×	Hybrid systems	×	Dendrimers on surfaces of metallic nanoparticles	[28, 47, 182]
×	×	×	Dendrimers on surfaces of ZnS/CdSe nanoparticles	[28, 43, 47]

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Chapter 5
The Dendritic State and Dendritic Effects

Before 1984, three classes of macromolecular architecture (i.e., linear, cross-linked, and branched) were widely accepted for construction of relatively polydisperse products of different molecular weights [13, 19, 32, 49, 57]. Since that year, the "dendritic state" is acknowledged as a new, fourth class of polymer architecture. It can be divided to five subclasses: random hyperbranched polymers, dendrigrafts, dendrons, dendrimers, and tecto(dendrimers) or megamers [9, 12, 16, 18, 19, 30, 32–34, 49, 51, 56–58, 60, 62]. Megamers are architectures/polymers [4, 23, 24] which were initially described as starburst polymers [61, 62] that are made using dendrimers as fundamental building blocks. They can be classified into two wide categories: statistical and structure-controlled megamers. In accord with Tomalia [49, 57] the precision architecture of dendrons/dendrimers allows these entities to be viewed as nanoscale monomer-type building blocks, which are suitable for the construction of regio-cross-linked dendrimers referred to as "megamers." The synthesis of dendrimers (in contrast to synthesis of "prior" macromolecules) proceeds smoothly to monodisperse, structure-controlled macromolecular systems similar to those observed in nature [1, 3, 8, 9, 12, 17, 25, 32, 34, 43, 44, 46, 51, 56, 66]. Starting from commercially available chemicals, dendrimeric polymers with polydispersities of $M_w/M_n \sim 1.0005$–1.05 are routinely obtained in multigram to kilogram scale [32, 34, 51, 56]. This was enabled also by simplifications of their synthesis by Lego-, ligation, and click chemistries [44, 49] (see also Chaps. 6.3, 6.4, and 6.5). Because of multistep dendrimer synthesis, the resulting dendrimer material is always a mixture of both ideal and nonideal structures [6, 9, 25, 43].

Dendrimers and hyperbranched polymers are partly overlapping groups [3, 17, 25, 43, 46, 60, 66], which differ in regularity of structure, synthetic availability, and chemical variability. In comparison with hyperbranched polymers, dendrimers are more regular and better defined, but more difficult to prepare (more expensive). It is very probable that the role of hyperbranched polymers will grow whereas the role of dendrimers will be shifted more to the field of fine chemicals. This book deals mainly with the topic of dendrimers with highlights of more important discoveries in the field of hyperbranched polymers.

J. Šebestík et al., *Biomedical Applications of Peptide-, Glyco- and Glycopeptide Dendrimers, and Analogous Dendrimeric Structures*,
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Due to the presence of a large number of terminal groups and the limitations or a complete lack of interpenetration, the physicochemical properties of dendrimers differ from those of classical polymers [6, 12]. The fundamental properties of dendrimers are [7, 8, 25, 49–51, 56, 58, 60, 63, 67]:

(1) The nanometer dimensions which are similar to the size of crucial biomacromolecules, such as proteins, and DNAs, which control the excretion from the body.
(2) Immense number of surface groups available for conjugation of signaling groups, targeting moieties, drugs, or biocompatibility groups. These conjugates can influence receptor-mediated targeting, therapy dosage, biodistribution, or controlled release of drugs from the interior space. As well, this conjugation augments or resists trans-cellular, epithelial, or vascular biopermeability.
(3) Conjugation of surface groups with PEGs leads in most cases to non- or low-immunogenic dendrimers.
(4) In the interior void space, small molecule drugs, metals, or probes can be encapsulated.
(5) The required biocompatibility is connected with lower generation anionic or neutral polar terminal surface groups in contrast to higher generation neutral apolar and cationic surface groups.

The above-mentioned dendritic properties are the consequence of the structure, the collective and cooperative actions of internal and external functional groups, the large number of termini, the high local concentrations of these internal/external functional groups, the ability to behave as dendrimer pseudophase, the soft to hard (organic) nanoparticle character, and the uniformity of molecules [6, 11, 32, 34, 37, 38, 49, 51, 55, 56, 58–60]. The number of terminal groups increases exponentially with increasing generations, together with the overall density of the dendrimer molecules. Inversely, the accessibility of all functional groups and flexibility of the dendrimer decrease. The increase of overall density and rigidity by high level of symmetry leads to limited interpenetration between molecules. When the density of terminal groups is too high, it limits the number of their intermolecular interactions and prevents the dendrimer penetration. The interactions occur on the surface of the dendrimer [6, 8, 36, 58, 60].

Generally, typical dendrimers [25] have a well-defined symmetrical structure, which contains 2–32 generations (G2–G32) with molecular weights from 2 to >100 kDa and diameters up to several nm. The size is therefore in the range of a medium-sized protein. In order to better understand the overall shape and structure of a glycodendrimer, MD (molecular dynamics) simulations have been used [27]. In spite of declarations suggesting that larger dendrimers should be good mimics for proteins, there are some important discrepancies. In comparison with proteins of comparable size, dendrimers are in general packed more loosely, while the density of functional groups on a dendrimer surface is much higher.

In comparison with other dendrimer types, the field of glycopeptide dendrimers was opened in 1993 by Roy [39–41]. The carbohydrate attached to the dendrimer surface was sialic acid that was introduced to confer strong inhibitory properties

against flu virus hemagglutinin, a lectin-like protein recognizing α-sialosides on respiratory mucins

The folding of dendrimers depends strongly on the used solvent. Solvents compatible with terminal groups lead to expansion, but they are not disentangled. In contrast, incompatible solvents induce burial of functional groups inside the dendrimers [6, 49, 50, 52].

The behavior of supramolecular systems including dendrimers can be controlled by pH. The understanding of this effect led to the construction of important molecular machines for electronic and biological applications [8, 26]. They can be regulated by simple perturbation with acids and bases.

Proteolysis strongly influences biological functions of proteins and peptides [48]. Branched peptides (MAPs) are resistant toward proteolysis by trypsin and α-chymotrypsin. The sensitivity of peptide dendrimers to proteases was controlled by the degree of branching. Dendrimers with two or more amino acids between branching points were easily cleaved by trypsin independently on the position of the reactive sequence within the dendrimers. On the other site, more compact dendrimers with only one amino acid between branching points were resistant toward trypsin cleavage. This topology controlled proteolysis provides a novel possibility for tuning of the biomedical properties of peptide dendrimers, which are not available in linear peptides. See also [10, 35, 36, 54].

In 2003 the Reymond's group reported a different type of peptide dendrimers. Their structure is based on classical MAPs [42, 53], but their functional groups, presented as amino acid side chains, were distributed throughout the branches [13–15] (Fig. 5.1). This design was later named "apple tree" configuration [36]. In classical MAPs, the functional groups are at the ends of the branches. Peptide dendrimers with an "apple tree" configuration have functional groups also in the branches, but they are not intended for branching but for selective introduction of special groups, labels, etc. These side chains can serve for providing specific functional groups (e.g., catalysis) or be chosen for fine-tuning of the physicochemical properties of the dendrimers including proteolysis. The "apple tree" configuration combines properties of organic dendrimers, e.g., dendritic effect and multivalency with properties typical for peptides and proteins, e.g., enzyme-like catalysis and susceptibility to degradation by proteases.

Dendrimers are able to form supramolecular structures via self-assembly [37,64]. These supramolecules play important role in molecular recognition. Because of the exponential growth of mass of dendrimer and only cubical growth of its volume with the number of generations, a maximum is present in the plot of logarithm of intrinsic viscosity versus molecular weight. Similarly, since intrinsic viscosity is inversely proportional to the density of solution, the solution density should display a minimum for maximal intrinsic viscosity. Minimal refractive index is characteristic for this point [6, 9, 50]. One obstacle of polyionic dendrimer pH titration is the separation of nanophases. Strong electrostatic repulsions of identical charges (protonated or deprotonated functional groups of dendrimer) are responsible for large structural reorganizations leading to minimization of energy. The reorganizations are accompanied with collapses or expansions of dendrimers leading to above-mentioned nanophase separations [6, 50, 51, 56].

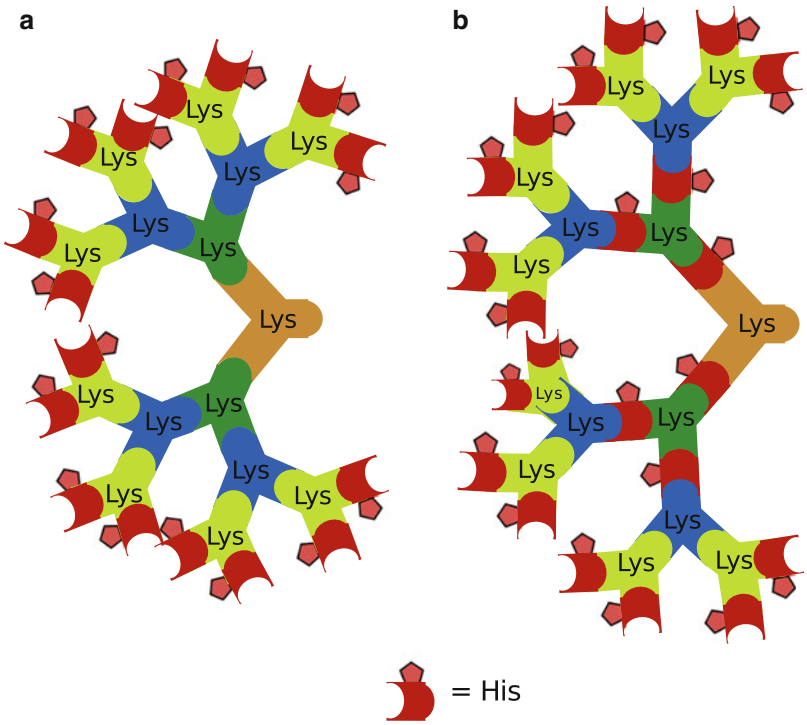

Fig. 5.1 Conventional MAP (**a**) versus "apple tree" (**b**). Adapted from [36]

Dendritic chain reaction (DCR) [45] represents a signal amplification technique that uses the disassembly properties of self-immolative dendrimers (Fig. 5.2). These self-immolative dendrimers liberate the end-group molecules by domino-like reactions which are activated by a single event. Because compounds released from the end groups gain the chemical reactivity for activation of another dendritic molecule, a capture of just one signal molecule leads to a chain reaction that disintegrates all of the dendritic molecules. The progress of this reaction is exponential, eventually releasing each of the end groups. This process with exponential amplification of signal has very high sensitivity and can be advantageously used for appropriate analyte.

The DCR technique was applied for detection of H_2O_2 by using AB3 dendron [45]. The dendron consists of a 4-nitroaniline reporter, two choline units, and phenylboronic acid as a trigger. The boronic acid reacts with hydrogen peroxide under mild alkaline conditions and generates intermediate phenylborate, which is then hydrolyzed to produce the corresponding phenol. This phenol further decomposes via several eliminations to 4-nitroaniline and the two choline molecules. The cleavage process generates one chromogenic reporter and two choline molecules. Choline oxidase transforms the two free cholines to betaines providing four

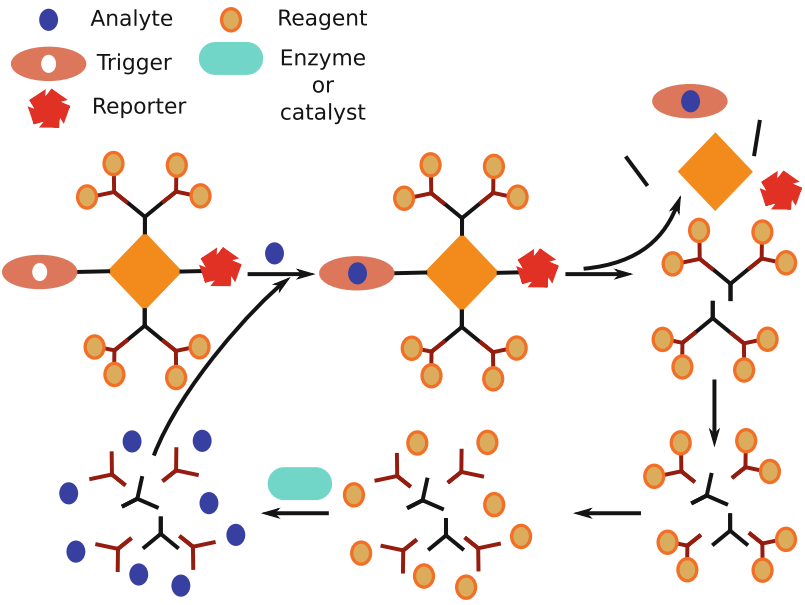

Fig. 5.2 Schematic illustration of dendritic chain reaction (DCR) [44,45]. After capture of the *blue* analyte by the trigger, the spontaneous decomposition of self-immolative dendrimer is initiated. In the course of decomposition, the reporter molecule, which can be detected via UV–VIS or fluorescence spectroscopy, is released. Another product of the decomposition is the reagent, which is converted to the analyte by the enzyme or the catalyst. This is the key step of the signal amplification. The figure was reprinted from [44] with kind permission of Springer, Wien–NewYork

molecules of hydrogen peroxide, which then activate additional four AB3 dendrons. The rate of the reaction should increase exponentially until each of the 4-nitroaniline molecules has been liberated.

The yellow color of the released 4-nitroaniline can be quantitatively evaluated. Owing to the amplification of background signal of spontaneous hydrolysis, the sensitivity of this system enables detection of analyte down to only 5 μM H_2O_2. Close to the limit of the assay sensitivity, the signal of the DCR technique was 53-fold stronger than that obtained without amplification. Because the DCR-based assay can be connected with another probe, detection of other analytes and biocatalysts is possible.

Significant differences exist between the structure of dendrimers in the solid phase and in solution. The reason is the removal of solvent molecules, leading to collapse of the spheroidal shape in the solid phase [6, 32, 50, 51, 56, 58]. Polymer-like glass transition temperatures (T_g) of these materials are observed during the first run of differential scanning calorimetry. This phenomenon is not observed on the consecutive runs. Extremely long annealing time is required, until the materials solidify and recover. In general, dendrimers are amorphous and do not form crystals,

owing to their symmetry and spherical shape. Their T_g depends on their family and generation and is not usually high. Their mechanical character can be improved by their conjugation into cross-linked polymeric systems. In molten state, dendrimers form ideal Newtonian liquids [6, 50, 51, 56, 58].

To reduce a dependency of the world on fossil-fuel energy, the idea to use hydrogels in order to replace petroleum-based plastics emerged. Preparation of a transparent hydrogel from water, clay (2–3 % by mass), and a very small proportion (0.4 % by mass) of organic components was described [65]. The hydrogels can be molded into free-standing and shape-persistent objects. When damaged they are able to rapidly and completely self-heal.

Two models are used to explain the chirality amplification in dendrimeric polymers by cooperative conformational equilibria: the "sergeants and soldiers" principle and the "majority rules" model [28]. The presence of small proportions of chiral monomers within a starting building block composed of mostly achiral monomers leads to the predominance of a single helical sense. Amplification of the small energetic preference by the rarity of helical reversals accounts for forcing the helix to adopt a uniform helical sense [28]. In the above "kickline" analogy, a chiral monomer which kicks in a fixed direction drives its neighbors to "kick" in the same direction to prevent destabilization due to mismatched kicking. Unwinding of formed helix is energetically disfavored. Thus this directionality propagates down the polymer and results in a large chiral amplification. Because a small number of chiral "sergeants" direct the conformational properties of a large number of achiral "soldiers," this phenomenon is termed as the "sergeants and soldiers" effect. Polymerization of a mixture containing enantiomeric monomers with small enantiomeric excess leads to dendritic polymer, which manifests a helical preference identical to the corresponding chiral homopolymer. Because this effect leads to chirality amplification of the major enantiomer, it is called the "majority rules" principle [28]. For more data see [2, 20–22, 29, 31, 47].

The propagation of a falling motion of one "dancer" throughout the row shows how the effect of a localized perturbation influences the conformation of macromolecular system. Further amplification is achieved by aggregation, which influences the helical reversals during the helix association. It shifts the helical equilibrium toward segments with higher helical bias due to their association in solution [28].

Incorporation of a single ferrocene unit in well-defined locations in dendrons was carried out and used for a systematic investigation of every layer of dendrimers [5]. In intermediate layers, the redox potential values of ferrocene were remarkably different from those at the core and the periphery. In spite of location dependency of redox potential values, no significant change in the rate of heterogeneous electron transfer (k_0) was observed with respect to locations. This was explained by the possibility to nullify the distance between the electrode and ferrocene unit by free rotation of dendrimer.

The topic of dendrimers in general, including their physicochemical properties and dendritic effects comprising several structure-controlled parameters referred to as "critical nanoscale design parameters," (CNDPs) was covered by excellent

theoretically oriented reviews [37, 38, 58–60]. These structure-driven parameters include (1) size, (2) shape, (3) surface chemistry, (4) flexibility/rigidity, (5) architecture, and (6) elemental composition. In analogy with Mendeleev's periodical table, a "nanoperiodic table" of self-assembled dendrons and supramolecular dendrimers allowing the prediction of the general features of tertiary structures from primary structures was elaborated [60]. The first examples of Mendeleev-like nanoperiodic tables have recently fulfilled these expected nano-property pattern/trend predictions. Prediction of the self-assembly patterns is possible in some cases with predictive accuracies of 85–90 % based on knowledge of the primary dendron CNDPs [37, 38, 58–60].

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Chapter 6
Synthesis of Dendrimers: Convergent and Divergent Approaches

Dendrimers are available by two major strategies (Fig. 6.1) [33, 37, 68, 113, 144–146, 179, 182, 186, 195, 200]. First, "the divergent strategy" is based on a growth of a dendron originating from a core site. In this strategy, monomeric modules are assembled in a radial, branch-upon-branch motif using certain dendritic rules and principles. The second method is a "convergent strategy." It begins from what will become the dendrimer shell inward to a reactive focal point, forming a single reactive dendron. A final dendrimer structure is assembled by reaction of several dendrons with a multifunctional core. Both strategies are significantly improved by "Lego," click, and ligation chemistries. They are described in individual sections. The synthesis of well-defined multivalent and multimodal dendritic structures and their use for biomedical applications has been reviewed [33, 176, 186].

By possessing major advantages such as cheap reagents, fast synthesis, exponential growth, and possibility to prepare large dendrimers, the divergent approach served as a guidance rule in dendrimer synthesis [5, 18, 30, 32, 33, 37, 41, 52, 99, 113, 119, 144, 147, 179, 181, 182, 194, 200, 211]. However, the divergent approach has also its downsides such as more complicated purification of synthesized compounds caused by the contamination of the product by deletion compounds with molecular weight, charge, polarity, hydrophilicity, etc. very similar to the desired product. Furthermore, the steric hindrance of bulky branches in higher generations of dendrimers suppresses couplings of next building blocks and is responsible for major defects on the dendrimer surface. Whether the cumulative defects of coupling failures has to be avoided, the purification of the growing glycodendrimer in every generation is necessary [5, 18, 30, 32, 33, 41, 52, 99, 119, 147, 179, 181, 182, 186, 194, 200, 211].

In summary, the divergent approach is an easy way for synthesis of dendrimers, especially when empowered either by chemical ligation or connection of carbohydrates and dendrimers with linking bridges.

The main advantages of the convergent approach are monodispersity of dendrimers, the accessibility of asymmetric dendrimers through an attachment of different types of dendrons to one core, and facile purification and characterization of the product [5, 18, 30, 32, 33, 37, 41, 52, 76, 99, 113, 119, 144, 147, 148, 155, 179, 181, 182, 186, 194, 200, 211]. The facile purification is based on significant

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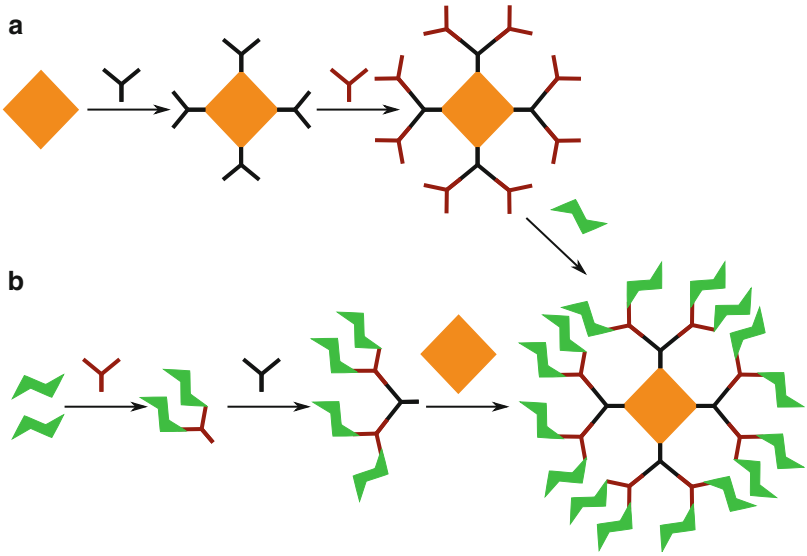

Fig. 6.1 Divergent (**a**) and convergent (**b**) strategies for dendrimer syntheses [32, 144, 179, 181, 182, 186, 195, 200]. The figure was reprinted from [186] with kind permission of Springer, Wien–NewYork

differences between the desired products and by-products differing by the lack of the entire branch. These differences in the molecular weight and charge are sufficient for successful separation of products and by-products by some of the techniques mentioned in the Chap. 7. Steric hindrances play a role of limiting factor during attachment of branches to the central core [5, 18, 30, 32, 33, 41, 52, 76, 99, 113, 119, 144, 179, 181, 182, 200].

Generally, many oligosaccharides, peptides, glycopeptides, linkers, and repeating units can be used as building blocks for convergent synthesis of dendritic structures providing a wide platform for the design of dendrimers with versatile properties. In many examples, the development of biologically active glycodendrimers by the convergent approach was successful. This overcomes the relatively low sugar loading efficacy in the divergent approach caused by the steric hindrance [119].

Andreu's team carried out comparative study between convergent (indirect) and divergent (direct) MAP synthesis [110]. In their direct approach, the dendritic construct with antigenic peptides was synthesized directly on solid support. This strategy needed careful optimization of all synthetic steps with introduction of hydrogen bond breakers. The indirect strategy synthesized the activated dendronized peptide and antigens separately. The segments are then purified and ligated together. The indirect approach was evaluated as less effective because the ligation of high excess of HPLC purified peptides is needed.

Hexaphenylbenzene was used as a rigid template for synthesis of star-shaped glycodendrimers [35]. Both convergent and divergent strategies were investigated. Glycocluster with up to 54 sugar ligands was obtained. Hydrophilic sugars counterbalanced hydrophobic moieties of aromatic core and provided water-soluble systems which can interact with lectins.

Selected examples of dendrimer syntheses by converged and divergent approaches are given below.

6.1 Divergent

An application of divergent approach is demonstrated on the syntheses of G0 to G5 monodisperse lysine MAGs [72] with benzhydrylamine core and 2 to 64 mono-, di-, and tri-α-mannopyranosyl residues on the shell. Mannosylating reagents based on active *N*-hydroxysuccinimide esters were used for derivatization of dendrimer amines in order to achieve complete glycation. According to RP-HPLC and MALDI-TOF MS, the purity was excellent. For visualizing of impurities, these methods are superior to NMR, especially for higher generation dendrimers. These MAGs induce insignificant dendritic cell maturation.

Another application of divergent approach was a direct and expedient synthesis of fluorophore-labeled MAGs [96] using commercially or easily available building blocks by automated Fmoc/tBu-SPGS. A large excess of reagents was used for driving the reaction to completion, thereby reducing imperfections within the dendritic structure. The strategy provided large, monodisperse G4 and G5 MAGs capped with 16 and 32 mannose residues, respectively, yielding [Man-α-O-$(CH_2)_3CO]_{16}$-$K_8K_4K_2$KF-$CONH_2$ (G4 glycodendron), and [Man-α-O-$(CH_2)_3CO]_{32}$-$K_{16}K_8K_4K_2$KF-$CONH_2$ (G5 glycodendron). A versatile fluorescence labeling of glycodendron was allowed by incorporation of a *C*-terminal lysine residue in the G4 MAG. The labeled MAGs [Man-α-O-$(CH_2)_3CO]_{16}$-$K_8K_4K_2$KK(FITC)F-$CONH_2$, [Man-α-O-$(CH_2)_3CO]_{16}$-$K_8K_4K_2$KK(pyrene)F-$CONH_2$, [Man-α-O-$(CH_2)_3CO]_{16}$-$K_8K_4K_2$KK(Dns)F-$CONH_2$, and [Man-α-O-$(CH_2)_3CO]_{16}$-$K_8K_4K_2$KK(Rhod)F-$CONH_2$ served for studies of carbohydrate–protein interactions by fluorescence spectroscopy and imaging methods. A pure and homogeneous compound was obtained by a single HPLC purification in all cases. The purity was confirmed by MALDI-TOF MS. The best probe for the imaging of mannose-receptor-mediated entry into dendritic cells by confocal fluorescence microscopy was the FITC-labeled G4 MAG.

Divergent synthesis of *N*-linked glycopeptides using solid-phase aspartylation were described [42]. The synthesis utilizes allyl ester protection of Asp side chain and Dmb protection of amide hydrogen in adjacent amino acids. The later protection is necessary to avoid aspartimide formation. After allyl removal on solid support, the coupling with aminoglycoside is carried out. Dmab ester was also investigated as an alternative to the allyl one. Since Dmab and allyl are orthogonal groups, two complementary glycosylation sites are available.

Divergent synthesis of glycopeptide dendrimers containing regions of MUC1 and MUC4 glycoproteins was carried out using MAP strategy on Wang linker [11].

Divergent synthesis of glycopeptide dendrons and subsequent conversion to dendrimers by disulfide bond formation was described for studies of recognition by Con A [59]. Homodimerization was considered as a convergent strategy. The homodimers inhibited Con A in enzyme-linked lectin assay.

6.2 Convergent

Glycopeptide dendrimer was synthesized by a convergent method (Fig. 6.2). Glycopeptide thioester R-Gly-Ser-Lys(R)-Ile-Leu-Leu-Thr-Ala-Ser-Leu- Asn(Man_3 $GlcNAc_2$)-Asp-Ser-Ala-Thr-Glu-Val-Thr-Gly-His-Arg-Trp-Leu-Lys(R)-Gly-SCH_2 CH_2CONH_2 (R = Boc) derived from extracellular matrix metalloproteinase inducer (emmprin) (34–58) was prepared by SPGS and ligated with an octavalent PAMAM dendrimer core possessing eight amino groups by the thioester method (2 equivalent to each hand of the dendrimer core) [155]. The final product was obtained after the Boc group removal by TFA. The glycopeptide dendrimer with an *N*-linked core pentasaccharide of about 30 kDa was obtained. The synthesis provided only a low amount of desired compound. Whereas side products can be hardly removed by reverse-phase HPLC, the SDS-PAGE provided sufficient resolution for removal of these impurities. The characterization was achieved by MALDI-TOF MS and amino acid analysis. Since the quantitative attachment of eight glycopeptide chains into the core was difficult, a new method has to be developed for a more efficient synthesis of larger glycopeptide dendrimers.

Another example of convergent approach is a synthesis of divalent and tetravalent polyether glycodendrons. The synthetic strategy cannot afford higher generation polyether glycodendrons. Nevertheless, the polyether glycodendron chemistry applied for incorporation of galactose moieties and for the synthesis of asymmetric glycodendrons was extended [56], including the scaffolding of both mannosyl and galactosyl residues. The focal point derivatization of these glycomimetics is necessary for biological applications.

Convergent synthesis of potential MUC1–lipopeptide vaccine was carried out using pentafluorophenyl ester-mediated segment condensation [219]. The vaccine candidate contained multiple copies of tumor-associated carbohydrate antigens (T_N and T) bound to lipopeptide immunoadjuvant $Pam_3CysSer$.

6.3 Lego Chemistry

Two sets of publications with intriguing concepts of “Molecular Meccano” [2, 3] and “Molecular Lego” were published. The first use of “Molecular Lego” was in Stoddart’s work [55, 107, 192] in 1988. Both concepts are the trade names of child’s toys in which complex structures are built from a limited number of simpler bricks.

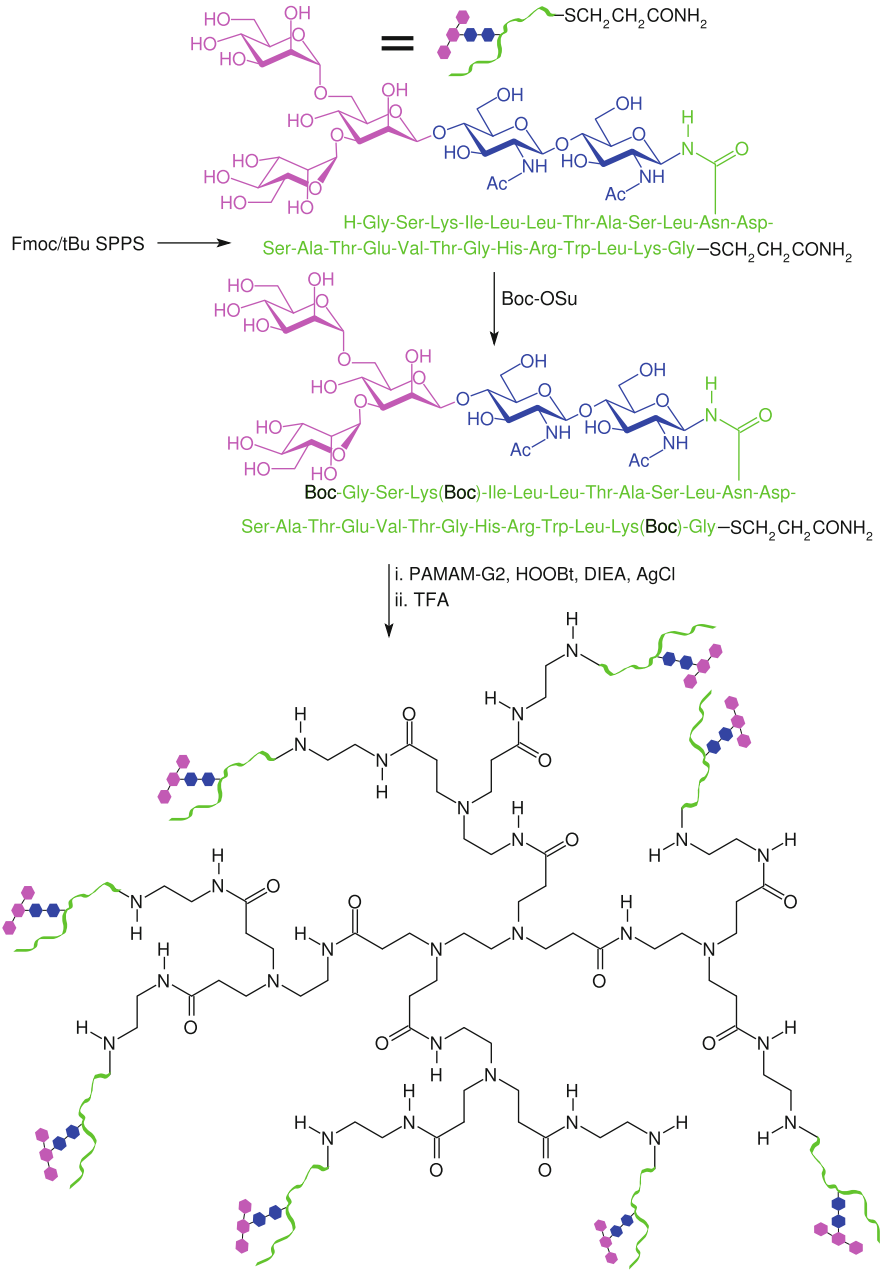

Fig. 6.2 Convergent synthesis of glycopeptide dendrimer using chemical ligation strategy [155, 186]. The figure was reprinted from [186] with kind permission of Springer, Wien–NewYork

Molecular Lego represents a basic set of small building blocks serving for the design and synthesis of nanoscale structures having virtually any shape and function desired [132, 183]. It relays on gluing of simple modules to each other in a facile manner [129] and it was applied for the preparation of macromolecules [19, 132]. The drawback of Lego strategy is a lack of immense variability of monomers ensuring regioselectivity of dendrimer forming reaction [211]. On the other hand, Lego is very powerful strategy for the rapid development of dendritic structures.

Lego chemistry was applied for the straightforward synthesis of dendrimers [127, 186, 194, 195, 211] and also for general organic chemistry [126, 132, 183].

6.4 Ligations

Chemoselectivity is a key feature of chemical ligations—special types of convergent synthesis. Chemical ligations are useful for coupling of non-protected peptides, glycopeptides, and other branches of dendrimers, using chemoselective condensation of amines, hydroxylamines, and hydrazines with aldehydes; reactions of aldehydes with vicinal amino alcohols and amino thiols; coupling of thioacids with alkylhalides; thioesters with vicinal aminothiols; phosphines with azides; and several reactions belonging to click chemistry, etc. (see Sect. 6.5). Chemical ligations have been extensively reviewed [1, 31, 33, 37, 51, 76, 103, 147, 160, 174, 186, 190, 209, 210], [26, 29, 49, 53, 70, 112, 115, 124, 148, 150, 173, 181, 182, 199, 203, 207, 211].

Imine ligations of peptide aldehydes and *N*-terminal Cys, Ser, and Thr provided chemoselectively pseudoprolines [57, 122, 123, 134, 197, 198]. A bidirectional mode for coupling of three unprotected peptides in tandem to form two pseudoproline bonds (thia- or oxaproline) was presented [134]. The condensation in the $C \rightarrow N$ direction is permitted by the chemoselectivity of sulfur of an amino terminal Cys over oxygen of a Ser or Thr peptide with a peptide that possesses a carboxyl terminal glycolaldehyde ester. A ligation in opposite direction was allowed by the masking of aldehyde function as glycerol ester. This tandem strategy is easily expendable for ligation of three or more segments. The pseudoproline formation was further carried out by others [208, 216].

Another chemoselective but reversible reaction is a reaction of the peptide aldehydes with hydrazines and *N*-oxyamines, which lead to corresponding hydrazones and oximes [21, 29, 46, 61, 66, 81, 94, 163, 178, 199, 223]. Lower pK_a values and good nucleophilicity make the hydrazones and oximes superior reagents over amines for reaction with aldehydes. Under slightly acidic condition, the amines are protected from the reaction by the protonation. The adducts of hydrazides and oximes can be stabilized by borohydride reductions [29, 199]. In order to accelerate the reaction of hydrazides and oximes with aldehydes, the aniline was used as a catalyst [46–48, 106, 117]. The catalyst is useful for synthesis of dynamic combinatorial libraries (DCLs). These reactions were applied for ligations of various peptides and proteins. Other examples are given [1, 29, 31, 76, 106, 117, 190, 199].

Two main drawbacks of previous ligation strategy (low stability of products and unnatural linkage) were cleared by formation of a quite stable amide bond. Ligation of protected peptides was carried out with 4-hydroxy-6-sulfanyldibenzofuran auxiliary [64,100–102]. First, the disulfide bridge, between the sulfanyl group of the *N*-terminal cysteine of one segment and the sulfanyl one belonging to the template at the *C*-terminus of the second segment, is formed in the sulfanyl-capture reaction. In the second step, the acyl transfer creates the amide bond between the two fragments.

Kemp's group started the investigation of the ligation of protected peptides [100, 102]. Later, probably inspired by Kent's publication of chemical ligation of unprotected segments in aqueous solution [184], Kemp also presented application of "prior-capture ligation" with unprotected segments in aqueous environment [101]. Interestingly, Kemp's "prior-capture ligation" provided natural amide bonds, whereas the Kent's first method [184] formed a less stable thioester related with the desired protein.

In 1994, the second method of Kent was presented [45] and named as "native chemical ligation." Similarly to the first method [184], which allows synthesis of the all-D-protein [103, 135], an enhanced nucleophilicity of sulfur is used. The first method uses the chemoselective reaction of alkyl bromide with sulfanyl group. A disadvantage of the method is a formation of an unnatural protein with thioester bond, which is not stable toward hydrolysis under physiological conditions. Second method ligates *C*-terminal thioester with *N*-terminal Cys and creates an amide bond to the Cys. The formation of the native amide bond paves the way for facile syntheses of proteins and glycoproteins [10,60,69,80,82,95,109, 120, 131, 140, 160, 188, 189, 201]. The syntheses of building bricks for the native chemical ligation can be achieved by both chemical [8, 17, 25, 27, 62, 73, 76, 82–84,91,104,118,133,142,152,169,185,187,196,212,214,225] and recombinant ways. The later ones applied intein-based synthesis of a protein thioester segment and specific proteases enhanced synthesis of the segment containing *N*-terminal Cys. The topic of expressed protein ligation was covered by many excellent reviews [10, 15, 44, 60, 61, 109, 120, 131, 138, 140, 160, 189]. Full convergence of the native chemical ligation was achieved by repeated ligations using both modular (*N*-terminal Cys protection) [6, 7, 103] and kinetically controlled approach [9, 17, 54, 103, 171].

The long-range ligation of *S*-acyl peptides with nonterminal Cys was accelerated by microwaves [77]. It is proceeding through macrocyclic transition states, which were favored in this order of their number of atoms: $5 \gg 14 > 11 \gg 8$.

Crucial limitations of the native chemical ligation are a low abundance of naturally occurring Cys residues [136] and high price of HPLC purified building blocks [186]. Several approaches were described to address the first issue, which can be divided to following groups: (1) an introduction of a cleavable/permanent auxiliary group on the α-amino group of *N*-terminal amino acid in the junction [75, 76, 95, 104, 160]; (2) an introduction of auxiliary sulfanyl group removable by reduction, acidolytic cleavage, or convertible by alkylation to amino acid side chain [43, 69, 74–76, 85, 95, 158, 160, 161, 171, 177, 213, 222]; (3) an introduction

of auxiliary sulfanyl group on a sugar moiety [12, 26, 76, 160]; and (4) a usage of partial protection of amino groups [39, 97]. For the second issue, see Sect. 7.1.

Removal of sulfur auxiliary after native chemical ligation by desulfurization was used for synthesis of glycopeptide analogues of fish antifreeze glycoproteins [69].

Native chemical ligations serve for efficient and systematic synthesis of a small glycoconjugate library containing human complex-type oligosaccharides [141].

Thiol auxiliary attached on saccharide moiety is extensively used in sugar-assisted ligations (SALs), which are powerful methods for the convergent construction of complex glycopeptides [12, 76, 160]. The influence of glycosylation at C-2, C-3, C-4, and C-6 on effectivity of SAL were studied. The investigations revealed that SAL is sensitive to extended glycosylation on the auxiliary-containing sugar. SAL is insensitive to extended glycosylation at C-4 and C-6. However, C-3 glycosylation completely suppressed the ligation reaction. Acceleration of the SAL with the substrate was observed for the glycopeptides containing up to six amino acid extensions at the *N*-terminus of the glycosylated residue [159].

Ligations of partially protected glycopeptide aryl esters of *O*-ethyldisulfanyl-phenol with/without *N*-cysteine glycopeptide were widely applied by Danishefsky's group [76, 95, 160].

An erythropoietin (EPO) analogue can cause cell proliferation at concentration in range of several pg per mL [80]. Glycosylated analogue of EPO with two human complex-type sialyloligosaccharides was accessed by a combination of chemical synthesis and protein expression. Analogue with glycosylated positions 24 and 30 had the same binding efficacy as the non-glycosylated material.

Native chemical ligation provided anti-inflammatory peptide-functionalized hydrogels [193]. These hydrogels encapsulated insulin-secreting cells and maintained their viability in the presence of a combination of cytokines such as TNF-α, IL-1β, and IFN-γ.

Ligation at Ser/Thr sites can be achieved by a thiohemiacetal auxiliary group [85]. After deprotection, the hemiacetal spontaneously hydrolyzes under mild acidic conditions.

For Ser junction, another possibility based on conversion to *S*-methylated Cys followed by CNBr cleavage exists [151]. The strategy provided sialylglycopeptide with 40 residues.

Modern ligation, which is orthogonal to aryl thioesters, leading to the native amide bond at Ser/Thr sites starts by capture of salicylaldehyde moiety of *C*-terminal esters with vicinal aminoalcohols of Ser/Thr [121]. In the equilibrium, the free amino group reacts intramolecularly with the acyl of *C*-terminal peptide. The auxiliary salicylaldehyde is cleaved under acidic conditions from unstable hemiacetal.

A new method for synthesis of a highly pure glycopeptide dendrimer based on the post-SPPS thioesterification was described [156]. The conversion of the stable amide bond to more labile thioester one was achieved by pH shift and *N*-alkylation. The equilibrium was further shifted with 3-mercaptopropionic acid. *N*-alkyl cysteine (NAC) at the *C*-terminus of the peptide played a role of such

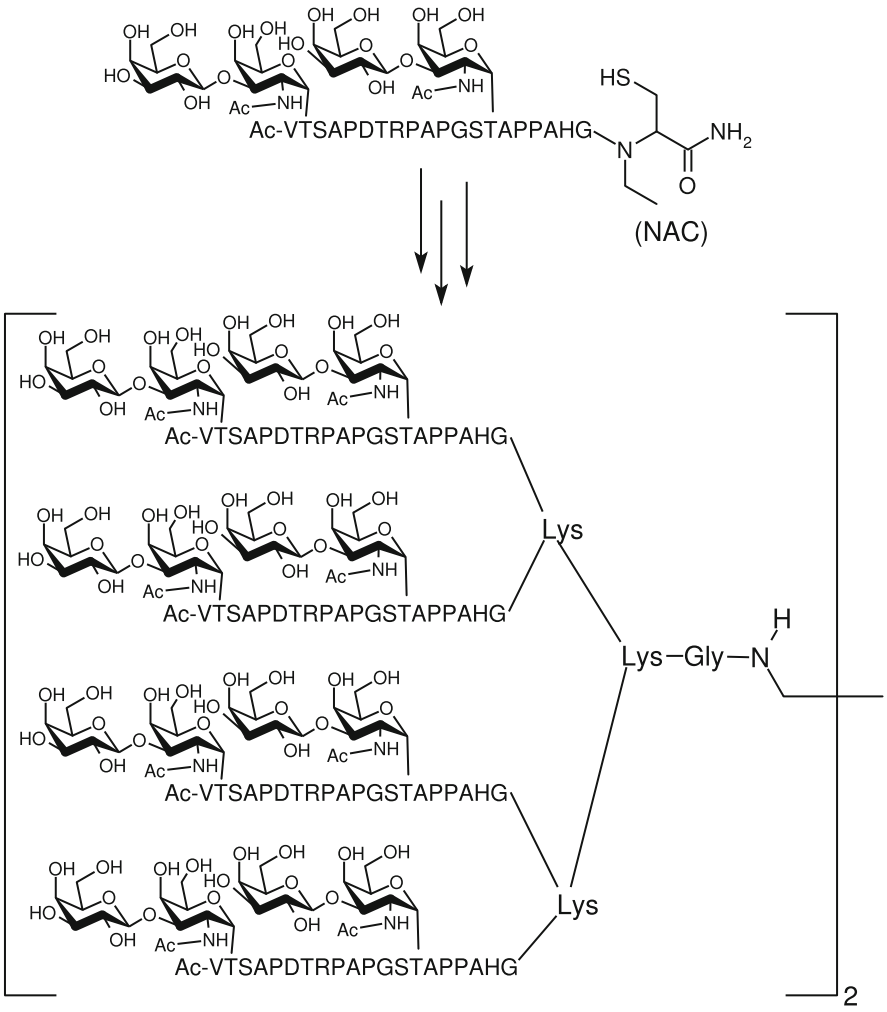

Fig. 6.3 *N*-alkyl cysteine (NAC)-assisted chemical ligation [156, 186]. The figure was reprinted from [186] with kind permission of Springer, Wien–NewYork

N- to *S*-acyl migratory device (Fig. 6.3). The principle was used for the synthesis of a glycopeptide dendrimer with eight peptide chains of the tandem repeat region of MUC1 containing two T-antigens. The highly pure glycopeptide dendrimer (22 kDa) was obtained using a sequential segment coupling assisted by NAC thioesterification.

Synthesis of NAC acyl migratory device directly on solid support was achieved recently [58]. Resin bound amine is sulfonylated with 2-nitrobenzensulfonyl chloride. High acidity of sulfonamides enables the selective methylation of Cys nitrogen.

2-Nitrobenzensulfonyl group is then selectively removed by 2-mercaptoethanol. This strategy was applied for convergent synthesis of ubiquitin thioester facilitated by photocleavable protection of previously mentioned NAC.

Design and synthesis of lipopeptide–carbohydrate assembled multivalent vaccine candidates was based on native chemical ligation [226].

Several other ligation techniques, such as His ligation [76], Staudinger ligation [29, 76], sulfo-click ligation [175], and enzyme-catalyzed ligation [13, 76, 130, 160, 167, 170], were also studied.

6.5 Click Chemistry

Click chemistry is one of the most promising strategies in dendrimer synthesis. Sharpless defined click chemistry as a type of reactions, which are modular, stereospecific (but not necessarily enantioselective), wide in scope, providing very high yields, and forming solely inoffensive by-products separable by non-chromatographic methods [108]. The system should be as simple as possible, ideally, insensitive to oxygen and water. The starting materials and reagents have to be readily available, and the process should require no solvent or a solvent that is easily removable. Simple product isolation is a must and purification has to be done by non-chromatographic or electrophoretic methods. Importantly, the product must be stable under physiological conditions [108]. Click chemistries [4, 16, 30, 32, 36, 37, 79, 108, 114, 115, 119, 124, 132, 143, 149, 182, 190, 207, 210, 211], [1, 49, 70, 86, 88, 113, 116, 165, 194, 195, 204, 215, 224] stand for group of reactions that are fast, ready to use, versatile, easy to purify, and regiospecific. Due to high selectivity, nearly perfect reliability, high yields, and broad tolerance toward many functional groups and reaction conditions, click reactions have been recently widely exploited for dendrimer and polymer synthesis, in pharmaceutical sciences, and for the modification of surfaces. Many examples and applications of click glycodendrimers were reviewed [1, 32, 33, 49, 67, 88, 115, 119, 164, 179, 182, 186, 195, 204, 207].

The high thermodynamic driving force ($\sim 20\,\mathrm{kcal\,mol^{-1}}$) is a reason for high stereospecificity and modularity of click reactions [143]. The click chemistry defines a synthetic concept or framework that comprises a range of reactions, with different reaction mechanisms, but similar reaction trajectories. Like many others, we have previously divided click chemistry into five main classes [49, 70, 79, 143, 182, 186, 207] and currently added one more [191]: (1) cycloaddition of unsaturated species: 1,3-dipolar cycloaddition; (2) cycloaddition of unsaturated species: [4+2]-cycloaddition (Diels–Alder); (3) cycloaddition of unsaturated species: [4+1]-cycloaddition (reaction of isonitriles); (4) nucleophilic substitution/ring-opening reactions; (5) addition to carbon–carbon multiple bonds; and (6) carbonyl reactions of the non-aldol type.

Owing to limited space and considering previous section about chemical ligations, we will restrict ourselves to the first type of reactions only, i.e., the role of Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition. The Cu(I)-catalyzed Huisgen

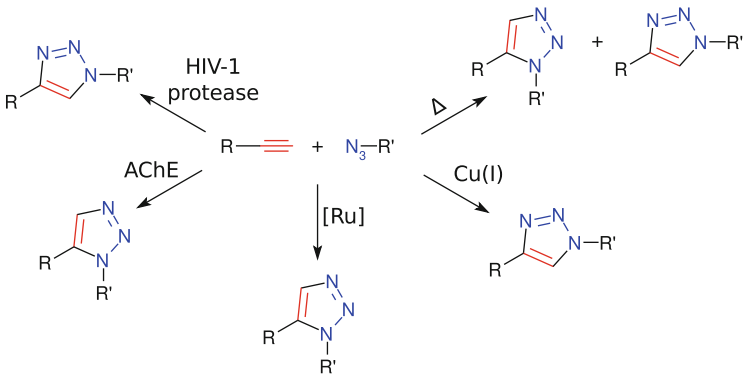

Fig. 6.4 Products of 1,3-dipolar cycloadditions between azides and alkynes [186, 207]. The figure was reprinted from [186] with kind permission of Springer, Wien–NewYork

1,3-dipolar cycloaddition of azides and terminal alkynes is a perfectly controlled reaction of multifunctional materials with tailor-made properties, especially dendrimers [37, 49, 70, 79, 143, 182, 186, 206, 207]. This cycloaddition is the most popular click reaction by far (Fig. 6.4). Click chemistry spread to a wide variety of disciplines, including dendrimers, polymer chemistry, material research, and the pharmaceutical sciences. Despite a wide scope of applications, there are some limitations for use of this cycloaddition such as toxicity of copper (a catalyst of the reaction) and of 1,2,3-triazoles (products). The Huisgen-type azide–alkyne cycloaddition catalyzed by Cu(I) is a powerful tool in new strategies for the synthesis of various types of densely glycosylated molecular architectures, such as glycoclusters, glycodendrimers, glycopolymers, and complex glycoconjugates.

As alkyne and azide groups can be a part of a wide range of compounds, the potential of this reaction is very high [86, 164, 207, 215, 217]. For nearly a half of a century, the reaction lacked a selectivity providing a mixture of the 1,4- and the 1,5-regioisomers [207]. Besides, this process depended on heating, required long reaction times for completion, and led to formation of the two regioisomers with consequences of laborious product separation using classical chromatographic procedures. Fortunately, two groups [180, 202] independently found that the addition of Cu(I) salts not only accelerated the cycloaddition reaction up to 10 million times but it also led to a regioselective formation of the 1,4-isomer (Fig. 6.4). The catalysis also reduced the reaction temperature to ambient level or just moderate heating could be required.

Another advantage of click reactions—sufficient reaction rate in highly diluted state—was demonstrated on synthesis of quatrefoil-shaped star-cyclic polystyrene containing a polyhedral oligomeric silsesquioxane core [71]. The synthesis was achieved by the combination of atom transfer radical polymerization (ATRP) and bimolecular click cyclization.

The use of the toxic copper catalyst can be avoided when strained alkynes, such as cyclooctynes, with conjugated electron withdrawing groups are used (Fig. 6.5).

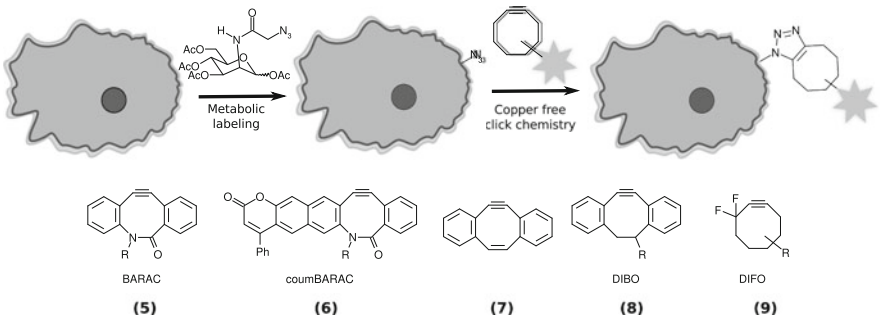

Fig. 6.5 Living cell labeling by copper-free click chemistry (adapted from [38, 89, 90]). After metabolic labeling with azidosugars, the cells are prone to copper-free click chemistry. The coumBARAC system becomes inherently fluorescent by formation of triazole ring during click chemistry. The other systems require conjugation with fluorophores

This was demonstrated on mono- or difluorinated cyclooctynes [92] (Fig. 6.5, compound **9**). The higher degree of fluorination the faster the reaction is. This strain-promoted cycloaddition proceeds very efficiently with high chemoselectivity even in *in vivo* applications. Therefore it perfectly fits to in situ cross-linking. Since no copper or ligand/base is required, this is only two-component system providing little extractable material. This work presents monitoring of the kinetics of an in situ cross-linking process using the azide antisymmetric FTIR stretch.

DIFO-FLAG was the most efficient probe for labeling of serum or tissue-resident glycoproteins in mouse *in vivo* [38].

Another example of copper-free click chemistry uses tricyclic cyclooctyne derivatives with heteroatoms, carbonyl group, and conjugated two benzene rings as electron withdrawing systems [90]. These aromatic rings and conjugated carbonyl not only reduced electron density on alkyne moiety but also increased strain energy. Since the carbonyl group is a part of an amide bond, the resonance structure of which contains double bond between C-N, the reactivity of BARAC cyclooctyne system (**5**, **6**) lays down between stable dibenzocyclooctynes (**8**) and unstable dibenzocyclooctenynes (**7**). The later one is more reactive than the previous one. Thus, BARAC possesses good stability with reasonable reactivity for click reactions. Moreover, BARAC compounds are more soluble in water than corresponding all carbon cyclooctynes. The biarylazacyclooctynone conjugated with dyes (BARAC, Fig. 6.5) serves for fluorescence imaging of azide-labeled glycans in living Chinese hamster ovary cells. The washing steps after conjugation with living cells can be omitted due to high signal-to-noise ratio of nanomolar BARAC-derived probe. Furthermore, the labeling requires only 30 min treatment of cells with the probe.

In order to avoid further derivatization of BARAC with fluorescence dyes, the aromatic system of BARAC was extended similarly to mimic fluorescence dye coumarin [89]. This coumBARAC increases 10 times fluorescence quantum yields after triazole formation (Fig. 6.5).

Another extension was achieved by an introduction of two hydrophilic polar sulfonic acid groups [65]. The labeling agent selectively reacts with extracellular azides and leaves the intracellular untouched.

Click chemistry was applied for the synthesis of not only glycodendrimers but also various nanoparticles with surface covered by glycodendrimers [128, 220] (see Chap. 12). Glycation of nanoparticle surfaces enhanced biocompatibility of nanomaterials. Kleinert et al. [105] carried out the synthesis of a broad variety of functionalized molecules for assembly on gold, providing the formation of biologically relevant self-assembled monolayers (SAMs) using a modular approach: either utilizing the click reaction in solution or directly by "click on SAM."

Propargylated pentaerythritol phosphodiester oligomers (PePOs) were synthesized using phosphoramidite with a DNA synthesizer. Conjugation with an azido L-fucose derivative was carried out by click chemistry using microwave activation [139]. These conjugates were active against *P. aeruginosa* (see also Chap. 15.3). Glyco oligonucleotide dendrimers were effectively synthesized by two successive 1,3-dipolar cycloadditions (bi-click chemistry) [166]. Because alkyne and azide functions cannot be simultaneously on the same substrate since they would participate in an intramolecular reaction leading to cyclization, alkyne and azide functions belonging to the same oligonucleotide must be introduced or generated successively in order to react specifically with azide and alkyne derivates by intermolecular cycloadditions. First, oligonucleotides bearing alkyne and bromohexyl (precursor of azide) functions were synthesized. Second, the cycloaddition was carried out with an azidocarbohydrate derivative, introducing the first type of sugar on solid phase. Third, the bromohexyl groups were converted to azidohexyl groups, which were finally clicked with alkyne carbohydrate derivatives to introduce the second type of carbohydrate on solid support or in solution. This microwave-assisted bi-click chemistry [166] is highly effective and useful for the synthesis of other heteroconjugates of oligonucleotides.

Other applications of microwave-assisted synthesis of click glycodendrimers were published [23, 24, 93, 165, 168].

Multivalent melanotropin ligands were prepared by click chemistry using a tetravalent scaffold [22] based on 2,2-bis(aminopropyl)glycine. Due to multivalent effect, the branched peptides were up to 350 times more active against cells overexpressing melanocortin 4 receptors than the monomeric peptide.

Another ligand of GPCRs receptors was loaded on PAMAM dendrimers by click chemistry [98]. Extent of dendrimer loading influenced dramatically ligand activity.

The Huisgen cycloaddition provided a series of large aromatic glycodendrimers containing 27, 81, and 243 xylose units on a surface [28] with the molecular weight up to 130 kDa for G3-243- sugar dendrimer. The chirality of sugar makes these glycodendrimers a good tool for enantioselective catalysis.

Click chemistry facilitated the synthesis of mannosylated (α-Man) glycodendrimers (the authors use the term neoglycoconjugates) with aliphatic-, aromatic-, and carbohydrate-centered architectures differing in structural characteristics, such as valency, topology, and nature of the linker [162]. The binding characteristics (IC_{50} values) of these glycomimetics toward Con A have been evaluated to provide

the structure–activity relationships. The same group [154] used a series of different click-based techniques for efficient synthesis of multivalent, structurally diverse, heterogeneous neoglycoconjugates (glycodendrimers). These methodologies are highly efficient and allow easy access to a series of α-Man containing glycodendrimers with different valency, nature of the constitutive sugars, nature of the scaffold, and length of the linker. Remarkably, the substitution pattern and the distance between the sugars are key parameters influencing the binding capabilities of these compounds toward Con A.

Click chemistry was used for synthesis of chiral glycodendrimer with binaftyl core and glucose shell layer [172]. Two consecutive click reactions were employed.

An efficient synthesis of fluorescent and non-fluorescent multivalent glycodendrimers was achieved by Cu(I)-catalyzed azide–alkyne 1,3-dipolar cycloaddition [153]. Well-defined glycopolymer, glycocyclodextrin, or glycocluster cores displaying galactose or lactose epitopes have been chosen. The click multivalent glycodendrimers act as synthetic activators mimicking the lipopolysaccharide in cell adhesion and stimulation of monocyte/macrophage cell lines. Gal- and Lac-containing glycocyclodextrins were used as glycodendrimers with the highest adhesion and stimulation capabilities. These click compounds activated monocytes/macrophages and also had a potential to become new therapeutics [153].

Structural diversity of phthalocyanine macrocycles [40] was enhanced by click chemistry.

Roy's team prepared a new family of glycodendrimer scaffolds containing 12 and 18 peripheral α-mannopyranosidic units using Cu(I)-catalyzed 1,3-dipolar cycloadditions of oligo-sulfurated dendritic scaffolds bearing alkyne groups and TRIS (tris(hydroxymethyl)aminomethane) derivatives [34]. A saturation of inhibitory potency was reached depending on dendrimer scaffolding generation. The limited surface of the dendrimeric particle played a role of constrains for displaying of individual sugars toward their protein receptors. Structures and valency are critical parameters for the optimization of binding interactions, especially for design of new compounds with therapeutic potential, notably for the inhibition of adhesion of *E. coli* toward urothelial infections and other mannoside-binding proteins [34].

Triazole glycocluster libraries [50] with up to four 1,4-disubstituted 1,2,3-triazole rings with *C*-linked glycosyl fragments were constructed on various scaffolds via multiple cycloadditions of suitably polyfunctionalized calix[4]arene, adamantane, and benzene derivatives with ethynyl and azidomethyl *C*-glycosides. The cycloadditions provided exclusively the 1,4-disubstituted triazole ring with yield up to 98%. Wide scope of click chemistry together with high degree of efficiency constitutes a simple and versatile means for the attachment of various sugars to polyfunctionalized substrates.

Other tetra- and hexavalent mannoside inhibitors of the pro-apoptotic, antiproliferative, and cell surface clustering effects of Con A were synthesized by click chemistry with pentaerythritol scaffolds bearing either alkyne or azide functionalities and by Sonogashira coupling using pentaerythritol scaffolds bearing either alkyne or p-iodophenyl functionalities [63]. Their interactions with membrane type 1-matrix metalloproteinase (MT1-MMP) in marrow-derived mesenchymal stromal

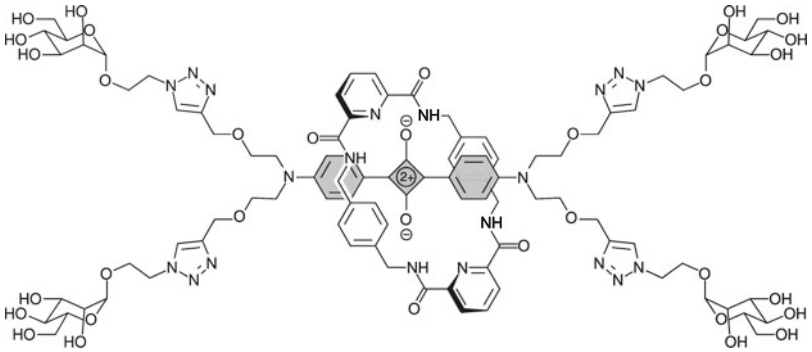

Fig. 6.6 Fluorescent squaraine rotaxanes obtained by click chemistry (Adapted from [221])

cells (MSC) were investigated. Con A-induced changes in MSC morphology were reversed by the tetra- and hexavalent mannosides. The mannosides are antagonists of Con A-induced caspase-3 activity and proMMP-2 activation. Antiproliferative and pro-apoptotic impact of Con A on the MT1-MMP/glucose-6-phosphate transporter signaling axis was reversed by these mannosides. Potential application of these mannosides in cancer therapy could be based on specific targeting of MT1-MMP pleiotropic functions in cell survival, proliferation, and extracellular matrix degradation [63].

The Sonogashira reaction was also used for synthesis of a library of 15 bivalent α-D-mannopyranosides with rigid linkers in order to evaluate the effects of inter-saccharide distances onto multivalent binding interactions with bacterial and plant lectins [14].

Several oligomannoside dendrimers with a 100-fold enhancement of affinity toward *E. coli* were synthesized by click chemistry using pentaerythritol scaffolds bearing either alkyne or azide functionalities [205].

Influence of linker obtained by Cu(I)-catalyzed click chemistry and by oxime ligation was compared [20]. Although no difference of binding was detected for the mannose-specific lectine, the compound carrying α-Fuc with oxime linkage possessed a significantly better affinity to the fucose-specific lectin.

Rotaxane glycodendrimers based on squaraine rotaxane scaffold were prepared by click chemistry [221] (Fig. 6.6). These amino-functionalized dendrimeric rotaxanes, which provide bright deep-red fluorescence, can be further derivatized. The dendritic shells with guanidinium, mannose, and phosphatidylcholine were investigated.

Promising strategy for synthesis of glycodendrimers was based on diazo transfer and click chemistry on solid phase [157]. Lysines are converted on resin to azides by Tf-N_3. It proceeds simultaneously with subsequent click chemistry. Even more powerful strategy for the diazo transfer reaction was described recently [78] using imidazole-1-sulfonyl azide hydrochloride.

Non-hydrolyzable dendrimer containing sialic acid derivatives was prepared by click cycloaddition [217] and evaluated as neuraminidase inhibitor. Micromolar IC_{50} value comparable to the known sialidase inhibitor *N*-acetyl-2,3-dehydro-2-deoxyneuraminic acid was obtained. Whereas the natural *O*-glycosides are vulnerable toward neuraminidase-catalyzed hydrolysis, the non-natural *N*-glycosides of sialic acid are resistant. These neuraminidase inhibitors can prevent a release of new virions.

A nice approach for tuning of biological properties is named "in situ click chemistry" [207]. It is based on dynamic combinatorial library method (see Chap. 8), i.e., instead of preparation of a compound collections and subsequent screening, a mixture of building blocks reacts in a tight reaction chamber—an enzyme (target), which serves as a selector of appropriate inhibitor by allowing its formation in an enzyme active site. At room temperature, the enzyme brings alkyne closer to an azide-containing molecule; this leads to a decrease of the high energetic barrier of the Huisgen reaction without copper catalysis and serves as an enzymatic catalysis. Reactants which fit correctly into the active site form new potent ligands of the enzyme (Fig. 6.4) [87, 111, 125, 137]. Potent inhibitors of several enzymes such as acetylcholinesterase (AChE) [111, 125], HIV-1 protease [218], matrix metalloproteinase [87], and carbonic anhydrase [137] were discovered by in situ click chemistry strategy. In the case of AChE [111, 125], the enzyme catalyzed the formation of 1,5-disubstituted triazoles, whereas HIV-1 protease favored 1,4-disubstituted ones [218]. The enzyme can not only accelerate the click reaction but also change its regioselectivity by choosing one of two pathways of the Cu-non-catalyzed reaction. Finally, the fact that click chemistry is a true interdisciplinary reaction must be highlighted, especially that click chemistry forms bridges between chemistry and biology (such as in the activity-based protein profiling assay) and can lead to tailored syntheses using biology [32, 79, 164, 186, 207].

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Chapter 7
Purification and Characterization of Dendrimers

Various methods of dendrimers characterization have been deeply reviewed such as NMR, electron paramagnetic resonance (EPR), mass spectrometry, UV–VIS spectrometry, time-resolved and nonlinear optical spectroscopy, laser light scattering, optical rotation, circular dichroism (CD), synchrotron radiation-based circular dichroism (SRCD), IR, Raman, fluorescence, X-ray diffraction, small-angle X-ray scattering (SAXS), small-angle neutron scattering, atomic force microscopy (AFM), scanning tunneling microscopy (STM), optical tweezers, transmission electron microscopy (TEM), isothermal titration calorimetry (ITC), different chromatographic and electromigration methods (electrophoresis, capillary electrophoresis), dielectric spectroscopy, differential scanning calorimetry, etc. [7, 10, 13, 23, 24, 27, 30, 38, 42, 52, 53, 57]. Hence, this chapter is focused only on some ways, which are important for dendrimer characterization. Their con and pros are clearly discussed.

Dendrimers can be characterized and purified by separation methods, as well as they can be employed as stationary phase and/or selection factors improving the efficacy of separation techniques [30, 50]. The two main classes of separation techniques are chromatographic and electromigration methods. Moreover, it is mandatory to use methods differing in the separation principle in order to check preparative efficiency by other complementary separation techniques, such as RP-HPLC and CE [10, 29, 42, 50, 57, 69]. Unfortunately, many researchers check the final purity after separation using the same technique. This violated the main principle of complementary characterization of separated product. Moreover, even combination of two complementary techniques is not always sufficient for removal of all by-products differing by only a single modification or deletion (the negligible difference such as missing one amino acid in molecules with mass above 7 kDa).

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7.1 Chromatographic Methods

Dendrimers were applied for improvement of stationary phase selector. Multivalency as one of main factors influencing the chiral separation HPLC was studied using enantioselective polymeric support with aliphatic dendrons [37]. The bigger distance of the selector from the support core, the more active it is. These materials provided effective separation of enantiomers. The symmetry of selector played crucial role for specific selectivity of the chiral stationary phase. Perfect dendrons prepared by convergent approach in solution were much better chiral selectors than those obtained by solid-phase divergent method.

In contrast to the chiral selector, dendronized stationary phases based on melamine had lower sequestration capacity for perfectly made dendrons prepared by convergent methods [1]. The sequestration capacity is increasing proportionally to the generation of dendrons.

Another example of stationary phase modification is usage of PAMAM-dendronized silica for size-exclusion chromatography [47].

Surface transformation of PAMAM dendrimers and the product quality can be easily monitored by ultra-performance liquid chromatography (UPLC) [11]. As expected, UPLC provides a vastly improved analytical method for the characterization of dendrimer polydispersity and variance in a typical surface modification in comparison with HPLC. In the presented example, the surface modification of G4 PAMAM-$(NH_2)_{64}$ dendrimer with biotin was studied. UPLC with increased average number of theoretical plates by a factor of 7 and reduced retention times of analytes by 36 % enhanced the resolution capability for discrimination of surface variances in dendrimers. Moreover, the reduced band spreading during the separation process maintained higher concentration of the analytes at the point of detection. This led to decrease of injection volumes and improvement of detection limit by a factor of 100, i.e., UPLC detection limit for G4, PAMAMs, was 1.69×10^{-12} mol. Thus, the UPLC outperforms HPLC in the detection, purification, and separation of unmodified as well as surface-modified PAMAM dendrimers.

Also, with certain limitations, the simple RP-HPLC method with UV detection can serve for quantification of peptide dendrimers. The technique was applied for assessment of skin permeation experiments [41].

Preparative HPLC can be also a shortcoming of MAP synthesis by conjugation approach [33]. The "expensively" purified peptide is conjugated with high excess (12-fold for tetravalent MAP) in order to achieve completion of linking. Hence, the direct synthesis with one HPLC purification of final product could be more competitive in MAP assembly than a convergent synthesis with several HPLC purifications.

For further comparison of HPLC and UPLC (robustness and ruggedness) see [16, 65].

Rapid and useful reaction monitoring by high-performance thin-layer chromatography (HPTLC) was described. The spot of separated compound can be characterized by MALDI-TOF-MS [8].

New fullerene glycodendron conjugates were prepared by the Diels–Alder reaction between C_{60} and anthryl glycodendron containing D- or L-gluconamides at the ends [62]. Moreover, two optically pure diastereomeric fullerodendrons were isolated from the mixture of diastereomers by silica gel column chromatography and GPC and characterized by ^{1}H and ^{13}C NMR spectroscopy, FTIR, and MALDI-TOF-MS analysis.

Average molecular weights of hyperbranched 5,6-glucan obtained by multi-angle laser light scattering (MALLS) were in range 7,400–122,400, in contrast to significantly smaller average molecular weight obtained by size-exclusion chromatography (SEC) [63]. These results when combined with viscosity measurements suggested an existence of these polymers in a compact spherical conformation.

Size-exclusion chromatography was found useful for purification of two glycocluster ligands with cyclam core bearing thiourea-linked glucose and 2-acetamido-2-deoxy-glucose at the periphery [61]. According to isothermal titration microcalorimetry, these glycoclusters interacted with Con A. Their stable complexes with the diagnostically relevant radioisotopes ^{99m}Tc and ^{64}Cu can be formed.

Gel permeation chromatography of sulfated oligosaccharide cluster with lysines revealed a narrow distribution with polydispersity index 1.08 [25]. The polylysine-dendritic sulfated cellobiose was analyzed by NMR and FTIR spectroscopies.

7.2 Electromigration Methods

Since capillary electrophoresis, capillary electrochromatography, and other electromigration methods are complementary to chromatographic methods [7, 10, 12, 29, 43, 46, 53, 54, 57, 58, 69], they can serve as independent purity assessment after purification of peptides, glycopeptides, and glycopeptide dendrimers by HPLC and UPLC.

Dendrimers play a dual role in electromigration methods, i.e., they are both the subject and object of separation [50]. For instance, dendrimers can serve as pseudo-stationary phase (PSP) for separation of other dendrimers [43]. When dendrimers served as modifier of the background electrolyte, higher performance of separation is achieved, in contrast to classical micellar electrokinetic chromatography [46]. The effect was explained by a higher homogeneity of the dendrimer phase and a broader migration time window [46]. Obviously, the separation was influenced by the size and charge of the dendrimers as well as by the composition of the electrolyte. The resolution is proportional to the concentration of dendrimer [43].

Both polyacrylamide gel (PAGE) and capillary electrophoreses (CE) were used for investigation of PAMAM dendrimers and their derivatives [9]. CE achieved separation up to the fifth generation of ammonia-core PAMAM dendrimers.

If the separation of negatively charged PAMAM succinamic acid dendrimers (PAMAM-SAH) (G1–G8) by CE was required, coating of capillary with a poly(vinyl alcohol) was mandatory [19]. The coating provided strong reproducibility of

separation for all generations of dendrimers. In contrast to amino-terminated PAMAM dendrimers, a reverse trend of migration for the PAMAM-SAH dendrimers was observed. The migration time was inversely proportional to generations. Due to this reverse trend, the separation of G1–G3 dendrimers was simple. Obviously, the same phenomenon disfavored a separation of higher generations (G4–G5).

Affinity capillary electrophoresis (ACE) can serve for determination of binding constants of vasoactive intestinal peptide to PAMAM dendrimers [21].

Separation of various amines was achieved by dynamic coating CE [51], where the common drawback of capillary zone electrophoresis (CZE) such as the sticking of these solutes with the capillary wall was removed. Dynamic coating CE separated PAMAM dendrimers of seven generations (G0–G6) at pH 7.4, in contrast to CZE. When the polyethyleneimine was used as a dynamic coating agent, the separation at acidic pH was improved.

Synthesis of G5 ethylenediamine-core PAMAM dendrimers with different degrees of acetylation and carboxylation was described [55]. They were investigated as models of the effect of charge and surface modifications of dendrimers on electrophoretic mobility (EM) and molecular distribution. Partial modification of dendrimers led to broader migration peaks than full modification or avoidance of modification. EM was inversely proportional to degree of surface acylation of both PAMAM acetamides and PAMAM succinamides. Due to nonlinear behavior of this phenomenon, the migration activity in CE separations can be hardly explained by charge/mass ratio changes.

The relative mobility of ethylenediamine-core PAMAM succinamic acid dendrimers was driven by molecular weight; hence, for the higher generation of dendrimers the slower migration was observed [59].

Comprehensive characterization of PAMAM dendrimer including the surface functionalization was carried out by Shi et al. [56]. G4 and G5 PAMAM dendrimers with acetamide, hydroxyl, and carboxyl surface group were synthesized, purified by dialysis, and characterized by PAGE, CE, SEC, MALDI-TOF-MS, and NMR.

Coating of capillary column with a carbosilane dendrimer was described [15]. The coating prevented the absorption of Si-O to basic substances such as adenine, adenosine, and 6-furfurylaminopurine. Their total separation was achieved within 20 min. Capillary after double coating provided the best separation effect.

The same laboratory [14] achieved chiral separation by capillary electrophoresis using column coated with carbosilane dendrimers with β-cyclodextrin. The analytes were chlortrimeton, promethazine, and benzedrine. The best resolution of enantiomers was observed for chlortrimeton.

Formation of stable nanoparticles between plasmid DNA (pDNA) and polycationic amphiphilic cyclodextrins served not only for the NA protection but also for separation of nucleic acids by gel electrophoresis [20].

7.3 Mass Spectrometry

For glycopeptides and glycopeptide dendrimers, the most important methods for structure elucidation and determination are mass spectrometries such as FAB-MS, MALDI-TOF-MS, ESI-MS, MS/MS tandem MS, and QqTOF quadrupole-quadrupole time-of-flight [10, 17, 18, 26, 34, 36, 39, 49]. These techniques provide the molecular weight of the product and even they can help in identification and quantification of certain impurities. Furthermore, MS techniques can describe conformation and dynamics space of biomolecules including glycopeptides [28].

ESI-MS methods were used for the determination of K_a values of anion during complexation by glycocluster thioureamethyl calix[4]resorcarenes [44]. It provided fast and quantitative characteristic of the complex formed between a host and a variety of guests.

Ionization of molecules is a hurdle of MS methods [50]. For some compounds, the correct masses were not provided by MALDI-TOF-MS spectra [4, 5, 42]. The results of the MALDI-TOF-MS depended on the molecular mass. With increasing mass (10.8–13.3 kDa), a significant difference was observed between the found (13,357.37 Da) and calculated (13,264.95 Da) masses [4, 5].

Another problem with ionization is observation of "ghost peaks" or "fake defects" [50] by both ESI-MS and MALDI-MS [6, 22, 49]. In the first case, ESI-MS analysis of PPI dendrimers presented a high occurrence of new type of defects, which were confirmed neither by ^{1}H, ^{13}C-NMR spectra nor by MALDI-MS. The second example describes opposite behavior of MALDI versus ESI. ESI-MS of sulfonamide rich dendrimers displayed the spectrum with high sample purity; however, MALDI-MS technique observed signals of defects that seem to be generated during synthesis. Finally, the thermal reactions during ionization within the matrix were responsible of their occurrence instead of synthetic problems. Therefore, mass spectra of dendrimers must be evaluated and interpreted with care, keeping in mind that false-negative and/or false-positive data can be obtained [42, 50].

MALDI-TOF spectra of G4 PEGylated and unPEGylated dendrimers provided mass with approximately 2 % deviations [31]. This relative deviation represents the absolute one around 300 Da.

Standard MALDI-TOF-MS provided masses of ultra-heavy compounds [40], such as immunoglobulin M and G10 PAMAM dendrimer.

Capillary electrophoresis with mass spectrometry was used for glycoscreening in biomedical applications [69].

Screening of molecular recognition and evaluation of self-assembly in supramolecular chemistry of dendrimers are possible by mass spectrometry and were extensively reviewed [48].

Amphiphilic dendrimers can form environment similar to normal and reverse micelles according to properties of the solvent [3]. Sequestration of a guest molecule was achieved by extraction, which was useful for separation of desired compounds. The assemblies mimicking reverse micelles were formed by these compounds with buried carboxylate groups—a hydrophilic and negatively charged interior—capable of sequestration of cations. It was an excellent tool for separation and

preconcentration of cationic peptides from aqueous solutions. The preconcentrated analytes can be directly detected by MALDI-MS. Moreover, the preconcentration amplified compound quantification and led to detection limits down to 500 pM from volumes as small as 250 μL.

During the synthesis of cell-surface epithelial glycopeptide derived from MUC1 by chemical ligation strategy the products and intermediates were characterized using ESI or MALDI-TOF-MS [68]. The increasing molecular weight of glycoconjugate caused an increase of mass spectrum background.

A correlation between degree of conjugation determined by NMR and laser-induced liquid beam ion desorption mass spectrometry (LILBID MS) was more successful than that between NMR and MALDI-TOF-MS [2].

CE with MALDI-TOF-MS was applied for development and validation of a complex dendrimeric contrast agent Gadomer [67]. CE provided separation of Gadomer 24 from related dendrimers and impurities of lower molecular weight. This served for the assurance and control of the quality of complex dendrimeric drug candidate Gadomer. MALDI-TOF-MS data were confirmed by measurements of complementary CE-ESI-TOF-MS [66]. Furthermore, high-resolution Fourier transform cyclotron resonance-MS (FTICR-MS) with/without CE led to mass spectra with the highest mass accuracy and resolution of several impurities presented in low concentrations in various Gadomer batches.

7.4 Miscellaneous Selected Examples

Combination of UV–VIS and EPR spectroscopies was used for investigation of metal–dendrimer interactions [2]. Metal binding of G3 to G5-PPI glycodendrimers containing either a dense maltose or maltotriose was generation dependent. Moreover, based on EPR analysis, internal and external Cu(II) coordination with axial and rhombic symmetry of the generated complexes was observed. External coordination of Cu(II) was facilitated by sugar groups and water molecules.

Electrochemical biosensor for glycated hemoglobin (HbA_{1c}) was developed using dendrimer and boronic acid as modifiers of electrode surface [60]. The detection of glycated hemoglobin was achieved in absence of expensive fluorescence probes and proteins such as anti-HbA_{1c} and haptoglobin. The boronic acid-modified electrode can function as a biosensor for clinical diagnostics of glycoproteins such as HbA_{1c}.

Photoinduced electron transfer from Ru(II) complexes to a quencher was used for design of fluorescence probe for lectins [32]. The sensor contained $Ru(bipy)_3$ core and a derivative of bipyridyl quencher with boronic acids. Lectin binding led to dissociation of carbohydrate boronic acid complex and restoration of natural fluorescence of $Ru(bipy)_3$ system.

Surface plasmon resonance (SPR) is suitable technique for competitive binding assays for recognition of lectin glycopolymer interactions [45]. SPR easily revealed

the availability and bioactivity of Man-modified polymers on the surface during binding of Con A solution.

SPR served for investigation of affinities of mannosylated pentaerythritol core based dendrimers to *E. coli* FimH [64]. Compounds with subnanomolar affinities were described.

Back-scattering interferometry (BSI) is a label-free technique for quantification of carbohydrate–lectin binding [35]. Interactions between saccharides and lectins were rapidly determined. Size-dependent adsorption of sugar-functionalized PAMAM dendrimers was observed and correlated with the expected density of lectins on the surface. The BSI is as sensitive as SPR and quartz crystal microbalance techniques; sometimes it is even better.

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Chapter 8
Dendrimeric Libraries

Due to low affinity of interactions between carbohydrate receptors and modified oligosaccharides, glycopeptides have been explored as alternative mimetics. Glycopeptides are superior ligands with higher affinity for a receptor than the natural carbohydrate ligands [31, 36, 37, 39].

Two of the ways for creation and selection of new leads are glycopeptide and oligosaccharide libraries, which have been reviewed [1, 5, 8, 9, 14, 25, 26, 29, 31, 33–37, 39, 40] for different glycotopes (T_N, TF, mannose, GlcNAc, L-fucose, etc.), resins (TentaGel, PEGA, POEPOP, SPOCC, etc.), and applications. The main goal for the synthesis of combinatorial libraries is the discovery of new drug leads. The libraries are accessible by synthesis in solution, on the solid phase, by enzymes, and in living cells [1, 25, 26, 29, 31]. This powerful technique can lead to the identification and analysis of complex interactions between carbohydrates and their receptors [31, 39].

A selection of inhibitors preventing biofilm formation of *Pseudomonas aeruginosa* [9, 17, 19, 21, 22] was achieved by the library approach. *P. aeruginosa* is a human pathogenic bacterium, producing a L-fucose-specific lectin, LecB, implicated in tissue attachment and the formation of biofilms. A 15,625-membered glycopeptide dendrimer library [21] with α-*C*-L-fucosyl residues at the *N*-termini was prepared and screened for binding to L-fucose-specific lectins. Selected glycopeptide dendrimer (L-Fuc-α-CH_2-CO-Lys-Pro-Leu)$_4$-(Lys-Phe-Lys-Ile)$_2$-Lys-His-Ile-NH_2 was a potent ligand against *Ulex europaeus* lectin UEA-I (IC_{50} 11 μM) and *P. aeruginosa* lectin PA-IIL (IC_{50} 0.14 μM). Twenty-five times larger library (390,625 members) of self-encoding glycopeptide dendrimer provided compounds disrupting established biofilms of the wild-type strain of *P. aeruginosa* and several clinical isolates [17, 19]. Further optimization of the structure activity relationships led to inhibitors of *P. aeruginosa* biofilm formation (L-Fuc-α-p-O-C_6H_4-CO-Lys-Ala-Asp)$_4$-(Lys-Ser-Gly-Ala)$_2$-Lys-His-Ile-NH_2 [17] and (L-Fuc-α-CH_2-CO-Lys-Pro)$_8$-(Lys-Leu-Phe)$_4$-(Lys-Lys-Ile)$_2$-Lys-His-Ile-NH_2 [22] with IC_{50} 0.11 and 0.025 μM, respectively. The last-mentioned compound was 440 times more active than L-fucose [22]. The fucosylated glycopeptide dendrimers are polyvalent inhibitors of *P. aeruginosa* adhesion and biofilm formation (Fig. 8.1).

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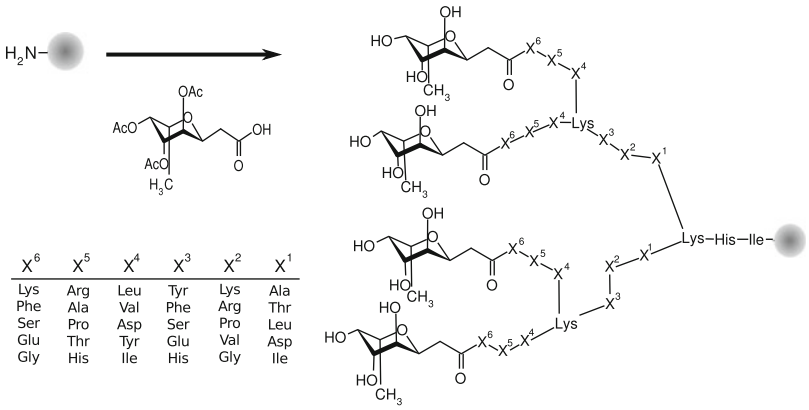

Fig. 8.1 Synthesis of the *C*-fucosyl peptide dendrimer library by split-and-mix SPPS on TentaGel resin [22, 39]. The figure was reprinted from [39] with kind permission of Springer, Wien–New York

A library of 15 bivalent α-D-mannopyranosides with rigid linkers was prepared [2]. The effects of inter-saccharide distances onto multivalent-binding interactions with bacterial and plant lectins were evaluated.

The *C*-linked α-galactosyl epitope mimetics with 2,3,4,6-tetra-*O*-acetyl-α-galactopyranosyl-(2-acetic acid) building block were screened with human anti-Gal Abs (IgG, IgM, and IgA), and finally MALDI sequencing served for identification of the mimetics of α-Gal epitopes [41]. A hit compound was a better inhibitor than those of known Gal-α-(1→3)-Gal peptide mimetics.

Microwave-assisted click chemistry using different poly-alkyne DNA-based scaffolds and two galactosyl azide derivatives [32] facilitated a synthesis of small libraries of di-, tri-, and tetragalactosyl clusters on solid support. The libraries were smoothly obtained with high purity suitable for direct biological evaluations.

Click chemistry enables the immobilization and synthesis of various dendrimers on the SAM, which provides glycodendrimer library array [13]. The libraries are well-defined saccharide arrays that can serve for several biological analyses such as profiling the saccharide-protein interactions and molecular and cellular proteomics.

Another lectin detection was based on digital analysis of Ru(II)-glycodendrimers fluorescence [20]. The fluorescent emission combined with logic analysis of the photoinduced electron transfer plays a role of a single-step and rapid method for screening of a glycodendrimer library.

Dynamic combinatorial libraries (DCL) emerged as very important approach for identification of new drugs [23]. The production of DCL in the presence of a target creates a library of compounds skewed toward structures that interact favorably with that target [15, 27, 30, 31, 36, 39]. The reactions involved in DCL formation must be reversible. DCL is a temporary pool of compounds that are reversible assemblies of building blocks. The final mixture contains the thermodynamically more stable product which is amplified in concentration by equilibrium-driven

selection process [23]. The equilibrium is shifted by binding of library members to a template. Strong binders are preferred instead of the weak ones. We can say that the newly created compounds can possess new or stronger biological activities than their "parents." The topic of dynamic combinatorial chemistry as a tool for the design of functional materials and devices was reviewed including many examples [7, 12, 16, 28]. The reviews and articles cited above have a general applicability and the principles described can be used also in the dendrimer field.

A topological cyclopeptide scaffold [11] was covered by oxime ligation strategy with biomolecules of various functionalities. Whereas imine or hydrazone linkages generate combinatorial libraries under thermodynamic control, the oxime bond is hardly reversible under ambient conditions. Thus, a synthesis of libraries from TASP and aminooxy building blocks should theoretically form a randomized and statistical distribution of each expected library species in a comparable amount—DCL. The proof of full randomization concept was carried out by SPR screening with a model lectin Con A [11]. It can be applied in glycomic or proteomic research for discovery of glycomimetics or selective ligands.

Self-replicating systems modeled an important role in the origin of life. DCL with 3,5-disulfanylbenzoic acid conjugated with several peptides was reported [4]. Replication of covalent structure was driven by nanostructure formation, caused by the assembly of the peptides into fibers held together by β-sheets. Mechanical forces were used as selector of the final covalent structure. The dominant product was chosen by selection of shaking or stirring. This is the first instance of an influence of yield of covalent synthesis by mechanical forces.

A delivery of exogenous DNA to human HeLa cells was achieved by a library of carriers based on various combinations of the cell penetrating peptide TAT, the SV40 large T protein nuclear localization signal (NLS) and a cationic dendrimeric peptide [42]. Chloroquine was used as a DNA protective agent enhancing transcription of nucleic acid cargos.

The synthesis, functional screening, and decoding of "one-bead-one-compound" (OBOC) libraries of dendrimers assembled from amino acid building blocks by "split-and-mix" SPPS were described [24]. A branching diamino acid attached at every third position provided the dendritic structure library with approximately 60,000 sequences. A restricted use of only four amino acids for splitting step served as unique encoding of the sequence of a dendrimer. The sequence was easily determined from an amino acid analysis of the solid support bead. In contrast to Edman sequencing, the amino acid analysis is more reliable, faster, and far less costly. The discovery of the catalysts for the hydrolysis of acyloxypyrene-trisulfonates was used as an example.

Dynamically formed glycopolymers by reversible acylhydrazone ligation [38] with inherent fluorescence may serve as biosensors of lectins, bacteria, toxins, viruses, etc.

Efficient catalysts for ester hydrolysis were based on histidine-containing dendritic polymer with general formula $(Ac–His\text{-}X)_8$-$(Dap\text{-}His\text{-}X)_4$-$(Dap\text{-}His\text{-}X)_2$-Dap-His-X–$NH_2$ [3]. After SPOT library selection, their catalytic proficiency was 2–3 orders of magnitude higher than that of 4-methyl imidazole.

Dendrimeric peptide inhibitors of human papillomaviruses infectivity were identified by minilibrary approach [10].

N-linked glycopeptides are available via solid-phase aspartylation [6]. This aspartylation will have far-reaching applications in the syntheses of *N*-linked glycopeptides and *N*-linked glycopeptide libraries.

OBOC glycopeptide dendrimer library with 65,536 members was tested for binding of Jurkat cells [18]. The identified dendrimer—(β-Gal-Gly-Arg-His-Ala)$_2$-Dap-Thr-Arg-His-Asp-Cys-NH_2—and its derivatives are efficient delivery vehicles. Its colchicine conjugate was cytotoxic with LD_{50} 1.5 μM, whereas the pentagalactosylated dendrimer—β-Gal_4-(Lys-Arg-His-Leu)$_2$-Dap-Thr-Tyr-His-Lys(β-Gal)-Cys-OH—selectively labels Jurkat cells, but its colchicine conjugate was not cytotoxic. Tubulin-binding assays demonstrated that the colchicine had to be released as the active drug.

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Chapter 9
Dendrimers in Catalysis

Dendrimer-encapsulated nanoparticles play a role of excellent catalysts for both homogeneous and heterogeneous catalysis [2, 21]. In catalytic applications, dendrimers serve as catalytically active species and/or soluble supports for catalytically active species [17, 18, 20]. Due to nanosize dimensions, dendrimers resemble biologically active enzymes with a little bit lower efficacy.

Small increments of free activation enthalpy govern enantioselectivity in a reaction. Thus, these transformations are in general suited for assessing "dendritic effects" which result from the immobilization of molecular catalysts. Chiral dendrimer catalysts are extremely selective due to a high level of molecular monodispersity, structural regularity, and well-defined catalytic sites. They were synthesized either by immobilization of chiral catalysts to achiral dendrimers or by attachment of achiral complexes to chiral dendrimer structures. The topic of stereoselective dendrimer catalysis was reviewed [10].

The combination of the advantages of both homogeneous and heterogeneous systems can serve as a Holy Grail of catalysis. Nanoparticles are leading heterogeneous catalysts with divergent reactivity and selectivity. A modification of heterogeneous platinum nanoparticles which can catalyze typical homogeneous catalytic reactions was described [24]. Platinum nanoparticles were selectively oxidized by the hypervalent iodine species $PhICl_2$ and catalyzed a range π-bond activation reactions. This modification of nanoparticles can serve for further development of reactions previously inaccessible in heterogeneous catalysis.

Chiral amphiphilic G1–G3 dendritic organocatalysts were synthesized [15]. Due to the presence of an optically active polar proline-derived core and nonpolar hydrocarbon dendrons, the organocatalysts were effective for the asymmetric aldol and nitro-Michael additions in oil-in-water emulsions. Larger hydrophobic dendrons improved the reaction enantioselectivity, promoted emulsion formation in water, decreased catalyst loading (to 1 mol%), and facilitated catalyst recovery after the reactions. On the other hand, larger dendrons increased the steric hindrance and lower catalytic activity. However, some of the G1- and G2-hindered dendritic organocatalysts possessed better catalytic activity. The dendritic catalysts can be

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recycled up to 5 times with negligible lost of product yield and enantiomeric excess. Moreover, cross contamination was not detectable even when the recovered G3 catalyst was reused in another reaction with different substrates.

A photooxidation of thioanisole by catalytically generated singlet oxygen ($^1O_2$) was reported [8]. As a catalyst, nanodevices with approximately 30 units of buckminsterfullerene (C_{60}) bound to a G4 PAMAM dendrimer was used. The reactivity was accelerated in aqueous solution, because improved diffusion of hydrophobic reactant molecules into dendrimer cavities.

As potential candidates for asymmetric or micellar catalysis, phosphorous glycodendrimers with hydrazone and xylose units have been studied [6]. These dendrimers can be both amphiphilic or hydrophobic according to end groups on shell sugar moieties.

Click nonaxylose dendrimers stabilized platinum nanoparticles and were catalytically active in olefin hydrogenation in water at room temperature [5]. Stabilizing Si-based dendrimers were prepared from cheap sugar—xylose—which is produced from corn. Water solubility of dendrimers and stabilized nanoparticles moved the olefin hydrogenation to neat aqueous environment without a requirement of organic cosolvents. The entire process became more "eco-friendly."

PPI dendrimers and hyperbranched PEIs were studied for copper-catalyzed hydrosilylation of acetophenone as soluble supports of carbo-BINAP ligands [11]. Whereas the PPI dendrimer contained up to 64 BINAP ligands per molecule, the hyperbranched PEI can have on average 9, 26, and 138 glutaroyl-AMINAP or carbo-BINAP ligands. The immobilized BINAP was recycled many times without any observable loss of activity or enantioselectivity.

Metal complexes of dendrimers were also used as catalysts for Wacker oxidation, hydroformylation, hydroxylation of aromatics, 2-naphthol coupling, and oxidative coupling of styrenes and benzene [9].

The 65,536-member library of peptide dendrimers was tested as hydrolytic catalyst of the fluorogenic substrate 1-butyryloxy-pyrene-2,7,8-trisulfonate and its derivatives [16]. Histidine catalytic activity at the dendrimer core was attenuated by anionic glutamate residues in the outer dendrimer branches.

The efficacy of artificial esterases based on polyhistidine peptides and polyhistidine peptide dendrimers was evaluated [3]. These catalysts provided cooperativity between multiple histidine imidazoles for binding and catalysis. The multivalent effects of hydrolysis—common property of peptide dendrimers—was also observed for linear peptides. Pre-equilibrium substrate binding was observed for the histidine tripeptide and longer oligomers. These electrostatic interactions multiplied the catalytic activity more than two orders of magnitude in comparison to reference molecule—4-methyl imidazole. The imidazole groups of the artificial esterases can act both as a nucleophile and general base. For 15 histidine units, dendrimeric esterases were approximately one order of magnitude more powerful than the linear equivalent. Further increasing of number of histidines did not provide a significant improvement of efficacy.

G4 hydroxyl-terminated PAMAM dendrimers were used as a template for synthesis of well-defined platinum catalysts dispersed in homemade silica

gel [1]. Nanoparticles with 40 Pt atoms served as an efficient catalyst for alkene isomerization from *trans* to *cis* isomers. Prior to this conversion, the nanoparticles have to be activated with H_2.

Transition metal nanoparticle catalysis in green solvents was reviewed [25]. Different sorts of dendrimers and their applications as homogeneous, heterogeneous, and solid-supported dendrimer catalysts were also reviewed [13]. For more details about use of dendrons and dendrimers in catalysis, see [2, 4, 7, 10, 12, 14, 15, 18–20, 22, 23].

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Part II
Dendrimers and Their Biological and Therapeutic Applications

Since particular dendrimeric structures are frequently designed and synthesized with a respect to their potential biological use, their biological functions have been touched in previous chapters primarily dedicated to their synthesis, structure, and physicochemical features. In the following chapters, the potential biological applications are discussed from the point of view of particular diseases and/or utilizations. Indeed, glyco- and glycopeptide dendrimers represent coding systems with extremely high density that can display enormous functional variability. The introduction of modern strategies of convergent glycodendrimer synthesis has led to immense growth of important discoveries. This paves the avenue for their applications as anticancer, antimicrobial, antiprion, and antiviral agents. They have been used as important tools in many novel techniques and biological applications, such as contrast agents for molecular imaging; drug and gene delivery; solubilization effect on drugs. They can serve as active components of novel vaccines, such as of tailor-made anticancer synthetic vaccines based on combination of small targeting peptides or tumor-associated antigens with dendrimeric cargos. Last but not least, neurodegenerative disorders (Alzheimer's disease and prion-caused diseases) seem to be inhibited with cationic glycodendrimers.

Chapter 10
Dendrimers and Solubility

Hydrophobicity of drugs and bioactive compounds reduces their solubility in water and significantly diminishes their activity *in vivo* [21]. Only 60 % of drug candidates reach the patients, whereas the remaining 40 % of compounds are eliminated during the screening owing to their low water solubility, poor bioavailability, and low permeability through biological membranes [1,14,22–25]. Therefore, a fundamental task of drug delivery is a thorough understanding of solubility enhancement. Dendrimers are a novel type of materials with a unique structure and properties which are suitable for drug delivery and solubilization.

Solubilization facilitated by dendrimers is achieved by ionic interactions, hydrogen bonding, and hydrophobic interactions. Dendrimers can have hydrophobic interior responsible for encapsulation of hydrophobic drugs and dendrimers can be covered by hydrophilic shell (e.g., carbohydrates) involved in interactions with water and solubilization [14, 22–24]. Modes of solubilizations are simple encapsulation (**10**, **11**), covalent conjugation (**12**, **13**, **14**, **16**), or electrostatic interaction (**15**, **16**) (Fig. 10.1) [21]. Dendrimer-based drug delivery not only improves the drug bioavailability, but also it provides protection of drugs against enzymatic or hydrolytic breakdown *in vivo* [23, 24, 26].

Dendrimers were applied as solubilizers of various drugs [21], such as anticancer drugs (camptothecin, carboplatin, chlorambucil, cisplatin, dimethoxycurcumin, doxorubicin, etoposide, 5-fluorouracil, methotrexate, oxaliplatin, and paclitaxel), antidepressants (venlafaxine), antifungal drugs (amphotericin B), antihemorrhagic drugs (nimodipine), antihistaminics (famotidine), anti-inflammatory drugs (diclofenac, diflunisal, ibuprofen, indomethacin, ketoprofen, mefenamic acid, methylprednisolone, naproxen, nifedipine, phenylbutazone, and piroxicam), and antimicrobial drugs (artemether, niclosamide, nadifloxacin, penicillin V, prulifloxacin, and sulfamethoxazole).

For instance, piroxicam-loaded G3 and G4 PAMAM dendrimers enhanced the drug solubility 107 and 222 times, respectively [19]. The half-life of elimination of the piroxicam conjugate was significantly higher than that of pure drug. G4-based conjugates were superior to G3 as well as pure piroxicam.

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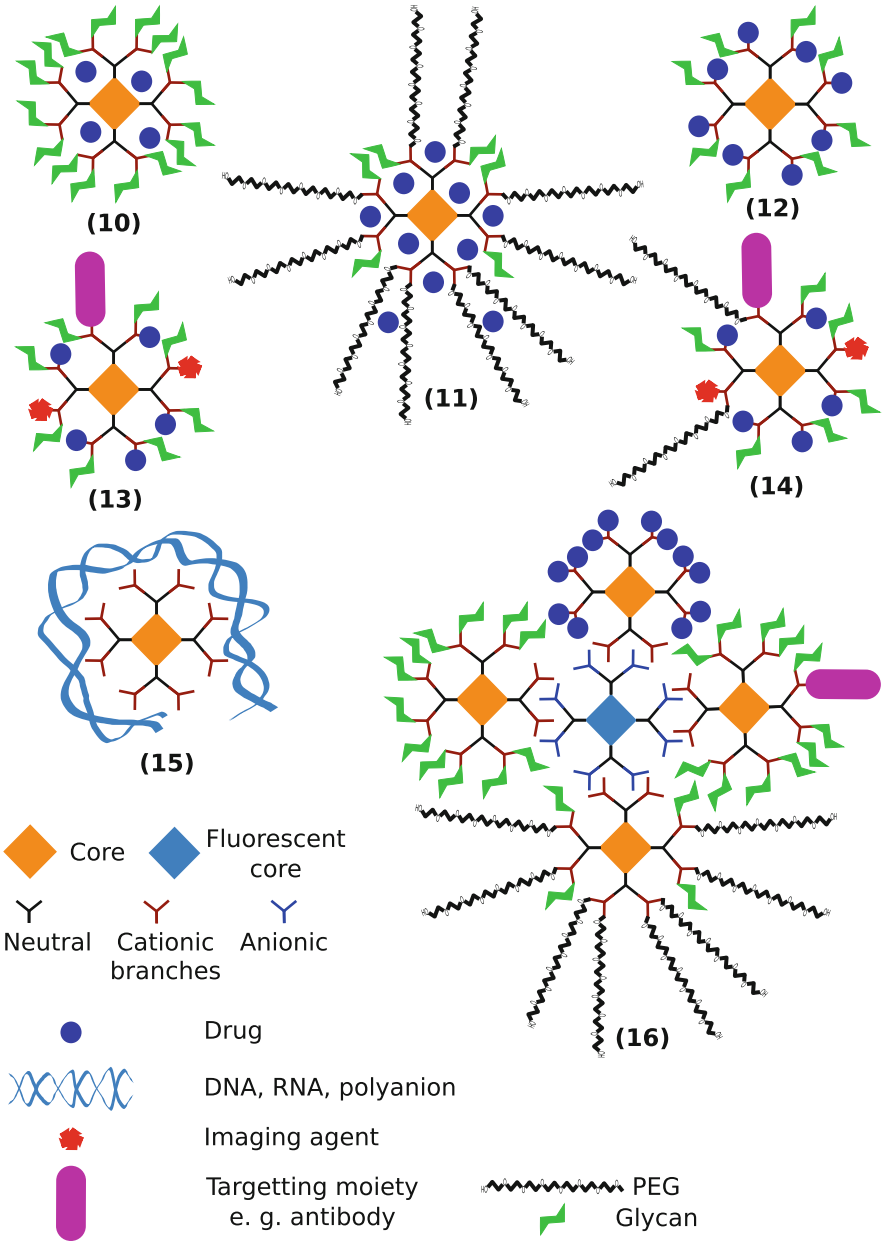

Fig. 10.1 Various ways of drug solubilization by dendrimers [21, 26]. The figure was reprinted from [21] with kind permission of Springer, Wien–NewYork

Another example is 1,000-fold solubility enhancement of the anticancer drug docetaxel [5] by the inclusion complexes of β-CD dendrimers with hexavalent mannosyl ligands.

The solubilization power of dendrimers can be improved by PEGylation [26]. A modification of PAMAM surface with PEG and β-CD enlarged the solubility of fullerene C_{60}—a unique structure with notable chemical and physical properties—by 18 orders of magnitude [17]. This example demonstrated how dendrimers can glue together extremely hydrophobic neat carbon with water.

PAMAM-b-poly(L-glutamate) dendrimers were synthesized using the ring-opening polymerization [20]. The critical aggregation concentration of the dendrimer was ruled by pH. The diameter of self-assembled nanoparticles was reduced by increasing pH of the solution. These self-assembled nanoparticles were nearly spherical. The response of nanoparticle assembly to pH changes was attributed to conformation transitions from α-helix to random coil.

Dendrimers with β-CD core were derivatized with 17-β-estradiol (E2) by click chemistry [16]. In cell culture, the dendrimer with four E2-β-CD conjugates was not cytotoxic and possessed all types of estrogenic activity such as binding to ER in the cytoplasm, causing ER dimerization, facilitating ER translocation to the nucleus, and transcription and expression of E2-responsive gene products. These effects were enabled by solubilization of hydrophobic estradiol derivative.

A brush poly(amidoamine) polymer was synthesized by copolymerization of 6-amino-6-deoxy-β-cyclodextrin (β-CDNH_2), 2-methylpiperazine, and 2,2-bis(acrylamido)acetic acid in aqueous medium [4]. These water-soluble and nontoxic polymers contain a plenty of β-CD groups capable to solubilize various drugs. A poorly soluble antiviral drug—acyclovir—served as a model for solubilization properties of the brush polymer. The brush dendrimer solubilized up to 11 % (w/w) of acyclovir. The rate of *in vitro* release of acyclovir is pH-dependent. The acyclovir–dendrimer complex had approximately 6-fold higher antiviral activity against herpes simplex virus type I than the free drug.

As a cheaper alternative toward dendrimers, the hyperbranched polymers are used for drug solubilization and delivery. A temperature-responsive hyperbranched polymer with a hydrophobic poly(3-ethyl-3-(hydroxymethyl)oxetane) core and thermosensitive poly(*N*-isopropylacrylamide) arms was prepared by the atom transfer radical polymerization [13]. The product self-assembled into multimolecular micelles with a diameter of 60 nm at room temperature. The micelles were loaded with a model drug-prednisone. Micelles entrapped about 31 % (w/w) of the drug with the entrapment efficiency fourfold higher than that of linear block polymers. This enhancement of entrapment was attributed to hyperbranched structure. At a lower critical solution temperature (LCST), these micelles have a reversible thermosensitive phase transition around 32 °C. For instance, at 23 °C, i.e., below the LCST, the drug release was slow and about 60 % of the drug remained buried in the micelle after 240 h. On the other hand, at 40 °C, i.e., above the LCST, the drug release was almost quantitative. This phenomenon can be applied for design of smart drugs which are released in the site of inflammation. Moreover, these micelles were not toxic toward HeLa cells.

Easy and systematic route toward new glycoclusters and G1-glycodendrimers with circular and aromatic polypropargylated scaffolds was described [6]. These systems containing up to 54 peripheral epitopes had a high valency. The hydrophobic character of the inner aromatic core was counterbalanced by the hydroxylated sugar derivatives, providing fully water-soluble derivatives.

Single-walled carbon nanotube (SWNT) is a nanocarrier of bioactive molecules in aqueous solution [12]. In order to improve SWNT solubility in water, the SWNT surface was modified with a series of dendritic β-galactopyranosides and α-mannopyranosides. Sugars with higher branching were more effective in solubilization of SWNT. G1 dendron solubilized the SWNT 3 and 4 times more than one corresponding sugar unit (Gal or Man, respectively). Functionalized nanotubes displayed multiple sugar units in pairs or quartets. These nanotubes can serve for sensing of pathogenic *Escherichia coli* and *Bacillus subtilis* (a nonvirulent model of *Bacillus anthracis* or anthrax).

Glycoclusters with pentaerytritol core and glucosamine hydrochloride in branches, as expected, not only provide the conjugated polymers with good solubility in water but also reduce the aggregation of polymers via the site isolation effect [7].

Easy method for preparation of water-soluble CdSe nanoparticles with diameters around 2 nm were described [10]. These CdSe quantum dots (fluorescence at 530 nm) were stabilized by G2-G5 PPI dendrimers containing maltose shell. The nanoparticle size was independent of dendrimer generation. The synthesis of uniform CdSe particles can be attributed to the formation of almost uniform spaces between densely packed glycodendrimers, which modify the nanoparticle surface. The surface modification stabilizes these quantum dots.

For more details about the influence of dendrimers on solubility, see [2,3,8,9,11,14,15,18,23–26].

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Chapter 11
Biocompatibility and Toxicity of Dendrimers

Closely related terms of toxicity and biocompatibility of dendrimers are important for biomedical applications [1, 3–5, 7, 24, 25, 27, 28, 31, 35]. Dendrimers should be devoid of toxicity and immunogenicity. The first concept—toxicity—is mainly applied by pharmacompanies for description of adverse effects to cells, organs, or patients. The second concept—"biocompatibility"—belongs to the field of biomedical materials as an extent of their compatibility with the studied system. One can say that biocompatibility and toxicity are inversely proportional, i.e., the more biocompatible the less toxic it is. Since 1986, biocompatibility is defined as the capability of a material to act with a proper host response in a specific application [7, 22, 25, 33]. Therefore, a biocompatibility of material can be described only if the precise context of material usage is known. Without knowledge of the exact usage of dendrimer, any chemistry cannot be defined as nontoxic or biocompatible. Clinical experience with dendrimers is still in its infancy; therefore, it is impossible to specify any chemistry insistingly biocompatible or toxic [7, 25].

The negative charge of most cell membranes interacts with amino surface groups of dendrimers. These electrostatic interactions significantly influence the stability and permeability of membranes [9, 12, 22, 25, 30]. The dendrimer–membrane interactions are also responsible for their high cellular uptake by endocytosis. However, G3 and higher generations of amino terminated dendrimers have a destructive interaction with the membrane, which causes cellular lysis and high cytotoxicity. Obviously, negatively charged dendrimers repulse with negatively charged membranes and therefore do not have generation-dependent cytotoxicity. For non-charged dendrimers, their cytotoxicity is influenced by polarity of surface groups. For instance, polar groups like PEG do not induce a toxic behavior. On the contrary, nonpolar groups like lipids could invade the membranes by hydrophobic interactions. This could result in dendrimer toxicity. Lipids can act as immunostimulators and positively influence the cells.

The particle size is a key player for biodistribution, clearance, and toxicity of dendrimers. The role of nanoparticles is summarized in Chap. 12.

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Inherent toxicity of PAMAM dendrimers, as well as their reticuloendothelial system uptake, and hemolysis restrict their clinical applications in drug delivery [35]. Due to positive charge and the surface size, there is a correlation between toxicological response of PAMAM dendrimers and the dendrimer generation [20, 21]. The higher the particle surface area the higher is the toxic response [25]. Interestingly, the surface modifications can modulate dendrimer toxicity. For example, PEG conjugation decreased the cytotoxicities and hemolysis of G5 and G6 PAMAM dendrimers [23]. Hence, desired biocompatibility correlates with lower generation of anionic or neutral polar groups, in contrast to higher generation of neutral apolar and cationic groups [25].

The spherical shape of PAMAM dendrimers can be responsible for their toxicity. Consequently, the asymmetric peptide dendrimers were designed [26]. Low generations of these asymmetric dendrimers exhibited minimal toxicity.

Brush dendrimers based on poly(*N*-substituent acrylamide)s with various alkylamine side chain were synthesized by free radical polymerization [14]. The dependence of toxicity on the methylene numbers in range from 2 to 12 was investigated. A trend of decreasing toxicity with increasing side-chain length was observed.

The development of a drug resistance is a common pitfall of many therapies. For instance, in the case of leukemia therapy, PAMAM dendrimers served for a dual drug delivery of antileukemic drugs [29]. Under optimized conditions, one molecule of PAMAM dendrimer entrapped approximately 27 and 8 molecules of methotrexate and all-trans retinoic acid, respectively. Release kinetics depends on the extent of protonation with an optimum at pH 7.4. On HeLa cell lines, dual drug-loaded dendrimer was more efficient than the free drug combination.

Low-molecular mass lysine-based peptide dendrimers interact with biological membranes and possess antibacterial activity [15, 16]. The dendrimers with protected ε-amino groups were the most toxic, whereas low toxicity was detected for dendrimers with protected α-amino groups. The steric distribution and type of hydrophobic groups and cationic centers influenced both toxicity and antimicrobial activity.

Coating of nanomaterials with glycodendrimers results in formation of biocompatible nanoparticles such as solid lipid nanoparticles [13], CNTs [11, 34, 36], fullerene C_{60} [17, 18, 25], lanthanide (Ln^{3+})-doped nanoparticles [2], and CdSe quantum dots [8]. These nanoparticles are not only biocompatible but also recognize specific lectins such as Con A, PNA, and PTA [2, 34].

For other chapters dealing with dendrimer toxicity and biocompatibility see [6, 10, 19, 32].

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Chapter 12
Dendrimers in Nanoscience and Nanotechnology

The word "nano(s)" means a dwarf in Greek. One nanometer corresponds to one-billionth of a meter, 10^{-9} m. This is approximately four and half times of the diameter of an individual carbon atom. Width of DNA is roughly 2.5 nm and size of protein molecules is in the range of 1–20 nm. Since nanotechnologies include the design, production, characterization, and application of structures, devices, and systems for management of size and shape at nanometer scale, they can handle and form materials at the atomic scale [42, 83, 88].

Nanotechnology provides useful materials, devices, and systems via the manipulation of tiny matter with at least one dimension smaller than 100 nm. Nanotechnology is an interdisciplinary science spreading to almost all hard sciences including physics, chemistry, biology, and medicine. Nanoscale devices are three to five orders of magnitude smaller than human cells. This means that their size corresponds to large biological molecules, such as enzymes and receptors, as shown in Fig. 12.1 [83, 88]. Their diameter in the range of 1–100 nm corresponds to molecular mass in the interval 10^4–10^7 Da and a number of atoms within 10^3–10^9 atoms [99]. These nanodimensions allowed nanoscale systems to act both on the cell surface and in inner parts of cells. Therefore, nanoscale systems can detect diseases and deliver the cargo to the exact target in a way unknown so far. The tailored artificial nanostructures can serve for sensing and repair of damaged parts of human body. In this way, their function can resemble or outperform naturally occurring biological systems, e.g., the white blood cells [88]. Due to nanoscale size of dendrimers, the multidisciplinary field of dendrimers and nanotechnology have many similar features and overlaps [5, 9, 16, 26, 42, 43, 49, 59, 63, 73, 76–78, 84, 87, 88, 90, 91, 93, 98–100, 108].

For decades, liposomes are used as carriers with unique properties such as a protection of drugs from degradation, site-specific targeting, and reduction of toxicity and other side effects. Unfortunately, their applications are limited because of inherent problems including poor stability and rapid leakage of water-soluble drug in the presence of blood components. These problems can be removed by substitution of liposomes with nanoparticles [87]. Nanoparticles not only increase

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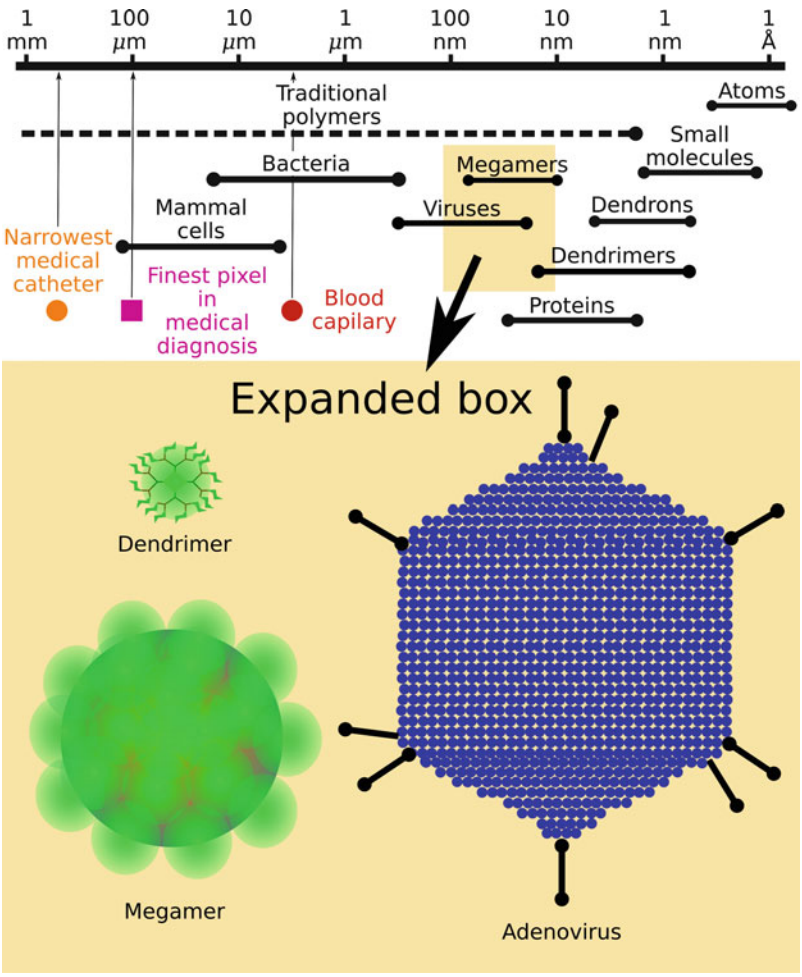

Fig. 12.1 Comparison of nano-, meso-, micro-, and macro-objects [83, 88]. The figure was reprinted from [83] with kind permission of Springer, Wien–NewYork

the stability of drugs–proteins but also provide controlled release of drug during delivery [42]. Among nanoparticles, drug-loaded dendrimers can serve as nanotransporters [76, 95].

Prefix nano is applied for many kinds of materials, technologies, and applications such as nanoarrays, nanobalances, nanobelts, nanobiotechnology, nanobridges, nanocatalysts, nanochannels, nanoclusters, nanocomposites, nanocrystalline materials, nanocrystals, nanocubes, nanodiamonds, nanodisks, nanodots, nanoelectrodes, nanoelectromechanical systems, nanoelectronics, nanoenvironments, nanofabrication, nanofibers, nanofingers, nanoislands, nanolenses, nanolithography, nanomanipulation, nanomaterials, nanomedicine, nanoparticle conjugates, nanoparticles,

nanoparticle synthesis, nanopatterning, nanophotonics, nanopillars, nanoplates, nanopores, nanoprinting, nanoprisms, nanorings, nanorods, nanoscale manipulation, nanoscience, nanoshells, nanoslits, nanostructured materials, nanostructured surfaces, nanostructures, nanotechnology, nanotips, nanotoxicology, nanotube binding, nanotubes, nanowire arrays, and nanowires. [1,6,8,9,13–17,19,21,22,24–27,29–32,34,38,42,44–46,50,51,54,56,59,60,64,65,68,69,71,73,74,76–78,81–83,85–87,89,92,95,97–102,104,105,108].

There is an overlap between sets of nanomaterials and dendrimers. The overlap can be seen in physical (size), chemical, and biological properties. In the following text we give some examples, where dendrimers (especially glyco and glycopeptide dendrimers) are both conjugated with other nanostructures or played a role of nanostructures themselves. The dendrimeric nanostructures possessed improved quality of physical, chemical, and biological properties such as solubility, stability, ability to work as delivery systems, and many others [1,6,8,9,13–17,19,21,22,24–27,29–32,34,38,42,44–46,50,51,54,56,59,60,64,65,68,69,71,73,74,76–78,81–83,85–87,89,92,95,97–102,104,105,108]. The immense potential of nanotechnology reaches to prevention, detection, diagnosis, and treatment of cancer, viral, and bacterial diseases.

"Nano" topics were extensively reviewed [1,5,6,8,9,13–17,19,21,22,24–27,29–32,34,38,42,44–46,50,51,54,56,59,60,62,64,65,68,69,71,73,74,76–78,81,82,85–87,89,92,95,97–102,104,105,108].

With increasing number of generations, the accessibility of dendrimer interior is decreasing. On the other hand, the persistency of the globular shape and rigidity of the molecules are increasing. The higher generation dendrimers resemble biomolecules, e.g., the accessibility of the surface group is higher than that of the interior ones [83]. The reaction of surface-exposed functions is faster, because the reagent can reach them directly, whereas it needs some time to diffuse into the interior. Another point of resemblance is caused by increased rigidity of the huge dendrimers which cannot penetrate each other. The limited interpenetration allows for surface interactions only and the dendrimers have to behave as individual organic nanoparticles. The organic nanoparticles under these restrains adopt perfectly symmetrical structure with extremely high relative density [7].

Dendrimers can be converted to nanodevices by covalent attachment of various molecules and building blocks. The nanodevice was defined by Balogh [7] as a polyfunctional nanoparticle capable of performance of various functions. The conjugation of dendrimers with various molecules provides many functions to nanodevices such as solubilization (PEGylation), regulation of charge (acetylation), light harvesting (conjugation with dyes), imaging (chelating ligands), and drug delivery (targeting sugars, peptides, or antibodies) [7,83].

Nanomaterials equipped with special chemical, optical, magnetic, and biological properties are requested by commercial or medical companies for cancer diagnosis [48] and drug delivery systems [41]. The properties of nanomaterial are easily tunable by selection of composition, shape, and size.

However, the same properties can be responsible for different reactions of human body than that of expected or desired ones for similar chemical compounds lacking

the nanodimensions. For instance, even a common element, such as carbon, can act differently in the body when it is introduced as a nanomaterial. This was illustrated on carbon nanotubes (CNTs) which cause cancer by the same way like asbestos [1, 47]. These primarily unexpected toxicity of some nanomaterials opened a social call for a critical look at nanotechnology and raised a questioning of common research methods and development of new ones for addressing key issues of the impact of nanotechnology on health and the environment [61, 83].

Owing to limited research data about nanomaterial toxicity, new ways of screening strategy should be pinpointed rather than a stepwise testing protocol. It is highly probable that the biological activity of nanoparticles depends on physicochemical parameters which are neglected in routinely performed toxicity assays. Hence, improper toxicity assays [47] can provide false-negative results in toxicity screening.

Coating of iron oxide nanoparticles with both dendritic and non-dendritic species provided mannose display on the surfaces and polymer vesicles for hemagglutination assay [56]. An advantage of dendritic functionalized vesicles and nanoparticles over non-dendritic ones is hemagglutinin-binding enhancement of 1–2 orders of magnitude. It was attributed to increased availability of the dendritic molecules on the surface because of their lower predilection for burial within the polymer coating. The limited interpenetrability of dendrimeric and polymeric material paws the way for enhancement of binding affinity of biological ligands anchored to polymer surfaces by dendritic scaffolds.

There are many biomedical applications widely using CNTs. Nevertheless, inherent cytotoxicity of CNTs is a limiting factor for even broader and safer use. The cytotoxicity of CNTs can be significantly suppressed by hydrophilic coating. One of these coatings was based on the glycodendrimers with 2,2-bis(hydroxymethyl)propionic acid building block [105]. A pyrene tail of the dendrimers is capable to bind single-walled CNTs (SWNTs) surfaces via π–π interactions whereas peripheral carbohydrate units of the dendrimers served for solubilization and biocompatibility of a final conjugate. This type of glycodendrimers with various carbohydrates were obtained by click chemistry in nearly quantitative yield. Formation of metastable colloid solutions was achieved by adsorption of the glycodendrimers onto SWNTs using ultrasonication in aqueous solution. Biocompatible solutions of glycodendrimer-functionalized SWNTs were stable for many months in water, whereas SWNTs lacking the functionalization precipitated within an hour. The development of new sensing and delivery systems requires specific binding of SWNT-bound glycodendrimers to receptors. This capability was addressed by a screening of a panel of (FITC)-conjugated lectins: Con A, *Arachis hypogaea* agglutinin (PNA), and *Psophocarpus tetragonolobus* agglutinin (PTA), which are specific for α-mannose, lactose, and β-galactose, respectively. Significant fluorescences were found during treatment of third generation of Man-SWNTs with Con A and Gal-SWNTs with PTA. As the terminal monosaccharide in Lac is Gal, the third generation of Lac-SWNTs responded to both PNA and PTA, but not to Con A. Interestingly, the cytotoxicity of SWNTs coated with third generations of glycodendrimer was completely attenuated in assay with

HEK293 cells. Glycodendrimeric coating prevents SWNT toxicity in smaller layers than those required for glycopolymeric coating [19]. Since glycodendrimer coating of SWNTs attenuated toxicity of nanotubes, the nanotubes can be applied for screening of various receptor interactions, receptor-based specific delivery of drugs, detection of carbohydrate-binding proteins, etc. [105].

It seems that the inherent cytotoxicity of CNTs can be avoided, when the carbon is substituted by boron nitride [20]. Boron nitride nanotubes (BNNTs) are nontoxic isosteres of CNTs with unique physical properties, thus, boron nitride forms stable hexagonal structure similar to the graphite one. Not only are BNNTs and CNTs structurally similar but also their thermal conductivity and mechanical properties are almost identical. In order to compare biological properties of CNTs and BNNTs, almost the same glycodendrimers were used for their coating as in the previous work [105]. Like CNTs, the G2 α-mannose-coated BNNTs are stable in aqueous solution for weeks, whereas the non-functionalized ones precipitated within 1 h from water. According to transmission electron microscopy (TEM), the studied multiwalled BNNTs were pure and regular with an outer diameter of approximately 20–30 nm and a length of up to 10 mm. Presence of an amorphous surface layer on TEM images served as a proof of BNNTs coating with glycodendrimers. As it was found for coated CNTs, the glycodendrimer-functionalized BNNTs interact with proteins as ligand with receptor, therefore nonspecific interactions with irrelevant proteins were not observed. The key feature of BNNTs is surface functionalization with biological epitopes capable of mediation of protein and cell binding. Functionalized BNNTs selectively delivered DNA oligomers to inner part of the cells without any apparent toxicity [20]. Hence, the biomedical applications of BNNTs could be superior to CNTs.

For other application of nanotubes see reviews [36, 110].

C_{60} fullerene plays a role of attractive spherical scaffolds—dendrimeric core—for anchoring of carbohydrates by very straightforward approach [79]. Sugars such as mannose can be attached by click chemistry onto a Bingel–Hirsch adduct of fullerene. Mannose presented on the spheric core was recognized by lectin (Con A) in multivalent manner. Using divalent dendron, the C_{60} fullerene can bear 24 mannose residues by one synthetic step of Huisgen click chemistry. Fullerene is an efficient platform for the globular presentation of carbohydrates for lectin–carbohydrate interactions providing a significant increase of affinity using a simple multivalent model.

Another straightforward modification of fullerene was achieved by Diels–Alder reaction [94]. Interestingly, the sugar dendron induced chirality of the fullerene–anthracene adduct (Fig. 12.2). The mixture of diastereoisomers was separated by column chromatography. The modified fullerene can be suitable starting material with inherent chirality.

For further reading about fullerenes see also [55, 67].

Self-assembled MAGs with up to 64 end groups of Gal-α-(1→3)-Gal-β-(1→4)-GlcNAc or Lac form non-covalent nanoparticles [96]. The nanoparticles, in contrast to the individual molecules, were efficient inhibitors of polyvalent interactions between IgM and the Gal-α-(1→3)-Gal-β-(1→4)-GlcNAc epitope, both *in vitro*

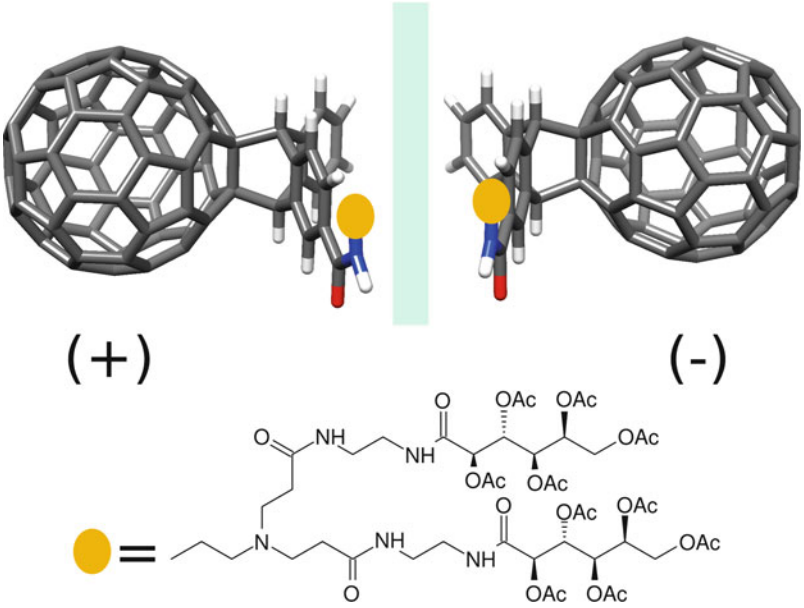

Fig. 12.2 Two diastereomers of fullerene glycodendron obtained by Diels–Alder reaction. Adapted from [94]

and *in vivo*. The activity of inhibitor was proportional to the size of the aggregates (nanoparticles) but not to the size of the individual molecules. Thus, the nanoparticles have different biological activity than was expected for individual molecules.

Artificial viruses and their aggregation were modeled with nanoparticles based on calix[4]resorcarene glycoclusters. Type of aggregation was dependent on the type of the saccharide attached to the calix[4]resorcarene scaffold. The degree of aggregation of artificial viruses decreased from highly aggregated α-Glc scaffolds through oligomeric β-Gal viruses to mostly monomeric β-Glc ones. The obtained nanoparticles were compactly packed, well charge-shielded, and transfected cell cultures (HeLa and HepG2) by a nonspecific but highly size-dependent endocytic pathway. The monomeric viruses were solely and highly active transfection agents [2–4, 10, 60, 64].

Synthesis of gold glyconanoparticles (GNPs) mimicking cells covered with glycosphingolipids membrane was described [75]. The GNPs were highly soluble under physiological conditions, stable against enzymatic degradation, and biocompatible. Since the initial step of tumor spreading involves tumor-associated carbohydrate antigens, GNPs coated with these antigens can inhibit experimental lung metastasis in a mouse melanoma model. Because significant suppression of metastases was observed for lactose-GNPs, the hypothesis of involvement of carbohydrate–carbohydrate interactions in the first step of the recognition process was corroborated. This clearly demonstrated a potential of Lac-GNPs for appli-

cation in biological processes with emphasis on anti-adhesive activity of GNPs suitable for suppression of tumor metastasis.

Guest–host nanodevices based on gold–PAMAM conjugates can serve for cancer imaging and therapy. This was proven on B16 mouse tumor model [25, 45, 66].

Inhibitors of DC-SIGN binding to gp120 were identified by library screening of multivalent water-soluble gold glyconanoparticles (manno-GNPs) presenting truncated $Man_9GlcNAc_2$. The best inhibitors were manno-GNPs with the disaccharide α-Man*p*-(1→2)-α-Man*p*. These GNPs were approximately 20,000 more active than the corresponding monomeric disaccharide [58].

Oligomannoside-functionalized gold nanoparticles inhibited the HIV trans-infection of T-cells mediated by DC-SIGN at nanomolar concentrations [57]. Hence, this carbohydrate multivalent nanoparticles form an anti-adhesive barrier during the early stage of HIV-1 infection. Interestingly, the presentation of simple linear di-, tri-, and tetra-oligosaccharides on the nanocluster provided efficiencies similar to those observed for complex-branched penta- and heptamannosides.

The synthesis of quite small and stable GNPs stabilized with hyperbranched polymers was achieved [109]. The hyperbranched multiarm copolymer of HBPO-star-PDEAEMA was prepared by an atom transfer radical polymerization (ATRP) method using a hydrophobic poly(3-ethyl-3-(hydroxymethyl)oxetane) (HBPO) core and many cationic poly(2-(*N,N*-diethylamino)ethyl methacrylate) (PDEAEMA) arms. The obtained GNPs were uniform in diameter (approximately 4 nm) and extremely stable in solution without apparent aggregation within 1 year. The pH of solution and ratio of ligand to Au could influence the size and stability of GNPs. The uniformity was attributed to two dominant properties of HBPOstar-PDEAEMA: self-reducibility and stabilization of GNPs. This method is useful for fabrication of GNPs in excellent quality and immense quantity (Fig. 12.3).

Another example of small gold nanoparticle synthesis was carried out by reduction of tetrachloroauric acid with glycodendrimers [72]. The nanoparticles with diameter between 1 and 2 nm were obtained by reduction with maltose-modified PPI dendrimers at high dendrimer generations (above G4). The lower generations led to bigger and polydisperse nanoparticles.

Water-soluble glycodendrimers containing cystamine were used for construction of coated gold nanoparticles (Au–man)[11]. These particles served for the detection of protein–carbohydrate interactions due to surface energy transfer process (SET). The SET is very sensitive technique useful for quantitative analysis of the binding constants of mannose-binding proteins.

A successful example of biphasic extraction system for efficient complex/covalent ligand exchange on the surface of CdSe/ZnS nanocrystals—quantum dots—was demonstrated [18]. The produced nanohybrids were highly water soluble with intact morphology and fluorescence properties of the nanocrystal core. This was achieved by solubilization with a highly hydrophilic dendron based on galactoside-capped gallamide with thiolate focal group. The nano-hybrid was smoothly and selectively internalized by lung cancer cells containing higher amount of membrane-bound sialoprotein receptors in 2–3 h.

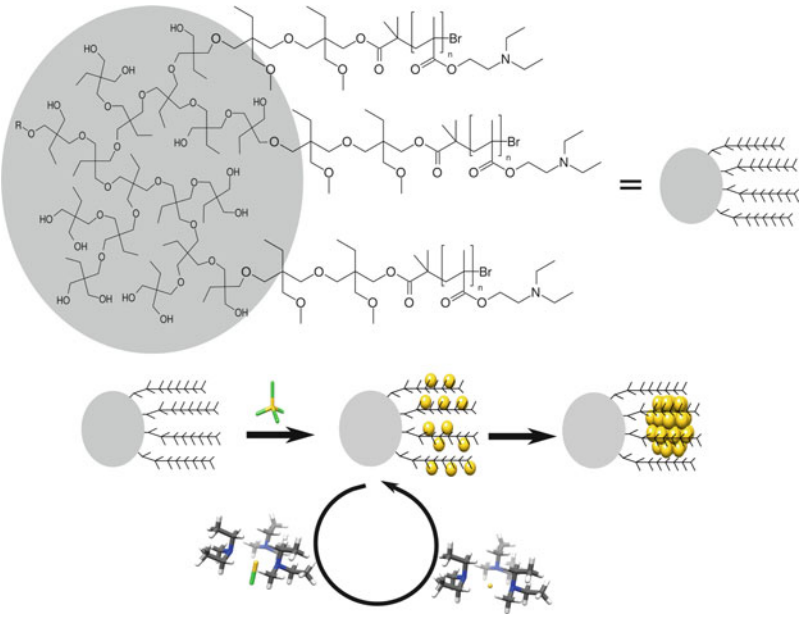

Fig. 12.3 Scheme of gold particle stabilization by hyperbranched polymers (Adapted from [109])

Water-soluble CdSe quantum dots were synthesized at room temperature by reaction of Cd(II) acetate with sodium hydrogen selenide in the presence of G2–G5 PPI dendrimers [28]. The formed nanoparticles had average approximately 2 nm in diameter, which is almost independent of dendrimer generations. The quantum dots fluorescence maximum was around 530 nm.

Lanthanide-doped upconverting nanoparticles are capable to convert near infrared (NIR) light to the visible one. Since a lack of autofluorescence, the sensors based on upconverting nanoparticles have high sensitivity of detection [37]. Due to higher penetration depths of NIR over VIS, the upconverting nanoparticles can be used for screening of tissues down to 10 cm under the skin. Their coating with carbohydrates makes them suitable probes for lectin-saccharide interactions acting by luminescence resonance energy transfer (LRET) [12]. PAMAM dendrimers were loaded on the surface of $NaGdF_4:Er^{3+}$, Yb^{3+} nanoparticles (LnNPs) by direct ligand exchange combined with subsequent linking with p-isothiocyanatophenyl α-mannopyranoside. The mannose-coated PAMAM-LnNPs were biocompatible, soluble in water, and recognized by Con A lectin. In order to achieve LRET, the Con A was conjugated with tetramethylrhodamine. The energy transfer from the Man-PAMAM-LnNPs induced by excitation at 980 nm to the labeled Con A was read as an increase of emission at 585 nm proportional to an increase of lectin concentration. In future, the sensors for pathogenic *Pseudomonas aeruginosa* and cancerous T-Ag receptors can be designed by coating of LnNPs with fucose and galactose.

PAMAM dendrons were used to increase the loading of immobilized enzyme—invertase—on the surface of magnetite nanoparticles [103]. In comparison with amino silanes surface modification, the load of enzyme was 2.5 times higher for a linker based on PAMAM dendrons. The stability of the invertase toward physical conditions such as temperature and pH was prolonged and enhanced by immobilization.

Adamantyl-functionalized dendrimers served as glue between β-CD SAM formed on the glass slide and on the polystyrene beads [52]. Interestingly, the supramolecular crystals held by non-covalent interactions were highly resistant to agitation by ultrasonication. These materials can be used as an ink for poly(dimethylsiloxane) (PDMS) stamps and can be printed onto a CD-functionalized surface. By a variation of the shape and size of the stamp, the printed patterns can form single-particle lines, interconnected particle rings, and V-shaped particle assemblies. The particles are artificial 3D receptors binding multiple complementary guest molecules. It means that the supramolecular host capabilities of the particle crystals were conserved during the fabrication process.

Ling and coworkers achieved a goal of nanotechnology, regenerability of surfaces, and reversibility of attachment of nanostructures onto them [53]. The goal was reached by the adsorption and desorption of β-CD-functionalized nanoparticles onto and from stimuli sensitive β-CD SAM containing preadsorbed ferrocenyl-derivatized PPI dendrimers (Fig. 12.4) [53]. Detachment of nanostructures from the β-CD SAMs was caused by electrochemical oxidation of the ferrocenyl end groups. Hydrophobic nanoparticles containing ferrocene remained strongly attached on the surface of the non-oxidized area, whereas, on the electrochemically oxidized area, complete removal of nanoparticles containing more hydrophilic ferrocenium cation was achieved.

Another application of host–guest interactions between β-CD and ferrocene-terminated PAMAM dendrimers was used for construction of molecular models on gold (111) printboards [33]. The simulation of surface coating with multivalent molecules was carried out by a divalently bound G0 ferrocene-terminated PAMAM dendrimer. Molecular dynamics (MD) simulations were examined in order to probe the dependency of printboard lattice constant, height, steric packing, hydrophobicity, and ink-binding properties on the gold-β-CD "linker" molecule and the degree of binding to gold.

Coarse-grained MD simulations of nanopatterning with multivalent inks based on β-CD SAMs were carried out by the same group [23].

A cooperative self-assembly of dendrons and CD attached on nanotubes and their nanotube–nanoparticle hybrids were used as "nanotube toolkit" [70]. The toolkit serves for sensing of proteins on the nanotube surface. The toolkit can be used as Lego with immense potential for the construction of functional nanomaterials.

Dramatical enhancement of dissociation constant between folate-binding protein and dendrimeric nanotherapeutics based on folate-G5 PAMAM dendrimer was observed [40]. The binding avidity was improved by multivalency up to 170,000 times. The paper corroborated hypothesis that multivalent enhancement of dissociation constant is responsible for increased biological activity instead of an accelerated endocytosis.

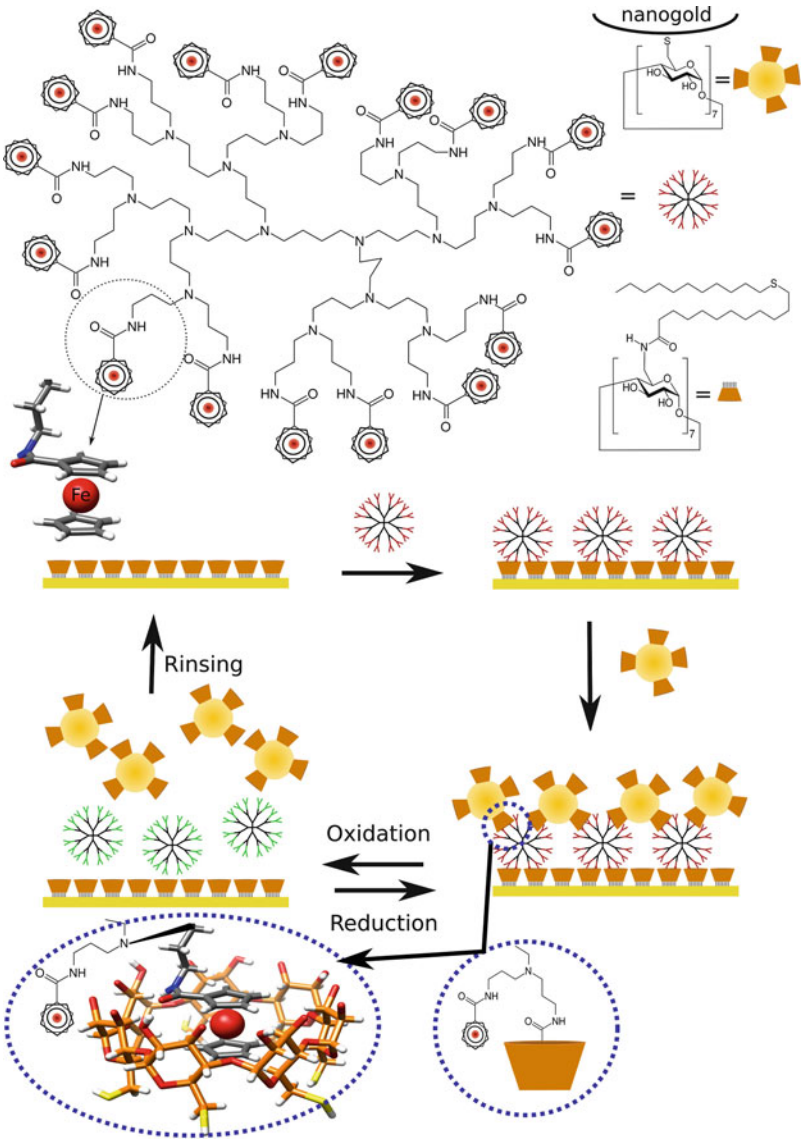

Fig. 12.4 A key goal of nanotechnology-regenerable surfaces and reversible attachment of nanostructures [53, 83]. The figure was reprinted from [83] with kind permission of Springer, Wien–NewYork

Palladium (PdNPs) and platinum (PtNPs) nanoparticles stabilized by water-soluble glycodendrimers were used for catalytic olefin hydrogenation in water at room temperature [35]. For further application of metal nanoparticles in catalysis see also Sect. 9 and the review [106].

Organic nanoparticles with strong photoluminescence were synthesized by Michael addition dispersion polymerization [107]. Their recognition by asialoglycoprotein receptors on the surface of HepG2 cancer cells led to internalization. Thus, the nanoparticles can be applied for imaging and specific delivery.

Amperometric sensors for glucose were based on conjugate of glucose oxidase with polyglycerol and chitosan dendrimers entrapped in polyaniline nanotubes [80]. A linear response of both sensors between 20 and 10,000 μM was observed. Polyglycerol-based sensor was more sensitive (∼ 25 %).

Another amperometric sensor for glucose was based on Pt nanoparticles encapsulated with a clay [39] with quantification range between 10 and 16,000 μM and detection limit 4 μM.

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Chapter 13
Dendrimers in Drug Delivery

There are three modes of drug delivery by dendrimers such as simple encapsulation, electrostatic interaction, or covalent conjugation [2, 6, 8–11, 16, 18, 22, 30, 36, 39, 42, 46, 50, 52, 53, 62, 66, 68]. The term "complex" is used for a dendrimer containing a free drug bound by non-covalent interactions and the term "conjugate" stands for a drug covalently attached to the dendrimer [46].

Owing to short clinical experience with dendrimers, a rational design of safe and nontoxic system is still not possible [52, 53]. Nevertheless, several examples of promising systems for drug delivery were already published.

Dendrimer-drug complexes and conjugates can be applied by any administration such as oral, intravenous, intraperitoneal, intratumoral, transdermal, and ocular. Drug-dendrimer conjugates possess two advantages over drug-dendrimer complexes and free drug dosing. First advantage is a prolonged lifetime of the drug in circulation. The second one is more stable level of the active substance preventing "local" drug overdose (Fig. 13.1) [10, 11].

Besides to their application as therapeutic agents, dendrimer– and polymer–drug conjugates together with other nanomedicines are increasingly studied also for diagnostic purposes. The combined use of therapeutics and diagnostics has resulted in the term "theranostics," defining delivery systems carrying both therapeutic and imaging or contrast agents [25, 30]. Such systems enable a more personalized medicine approach, in which therapy can be directly monitored and custom tailored.

Dendrimers have been also investigated for their capacity for tissue-specific drug targeting. Dendronized conjugates have been investigated as a potential platform for a development of compounds displaying relevant biological functions combined with a strong specific binding that could become an alternative for antibodies in diagnostic and therapeutic applications [37]. PAMAM dendrons were synthesized with c(RGDyK) peptide to create a scaffold for specific cell targeting and multivalent binding. Binary dendron-RGD conjugates were synthesized with a single Alexa Fluor 488, biotin, methotrexate, or additional functionalized dendron at the focal point. These complexes were capable to bind $\alpha V\beta 3$ integrin and thus

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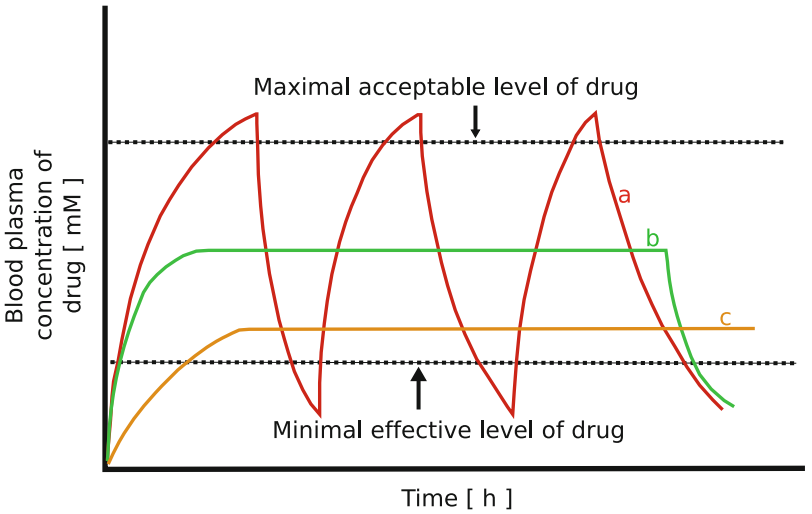

Fig. 13.1 Pharmacokinetics during therapy [10,11,53]. (**a**) Traditional drugs. (**b**) Drug-dendrimer complexes. (**c**) Drug-dendrimer conjugates. The figure was reprinted from [53] with kind permission of Springer, Wien–NewYork

specifically target active drugs to human umbilical vein endothelial cells (HUVEC) and human glioblastoma cells (U87MG).

In nanotechnology, an interest in development of new or improved antitumor drugs is increasing [34]. Some nanomaterials, such as fullerenes and their derivatives, have a potential to interact with the immune system and can be used as anticancer drugs. Other nanomaterials possess a capacity to deliver target drugs to cancer cells. Selective or enhanced transport of nanoparticles can employ unique conditions in tumor microenvironment, e.g., its enhanced permeability, retention effect, and the specific acidic conditions. Multifunctional and multiplexed nanoparticles represent next generation of nanoparticles under intensive investigation (see also Chap. 12).

For a better treatment of liver cancer, there is a need to increase a specificity of the chemotherapeutic agent delivery into hepatic cells. This could be achieved by utilization of asialoglycoprotein-mediated endocytosis. For this purpose, G5 PAMAM-NH_2 dendrimers were coupled with GalNAc via peptide and thiourea linkages [38]. Specific uptake of these complexes using asialoglycoprotein-mediated endocytosis was demonstrated in HepG2 cancer cells.

Recently, a targeted chemoimmuno drug-delivery system designed for a prostate cancer treatment has been developed [31]. A complex, consisting of G4 PAMAM-succinamic acid dendrimer and a plasmid-bearing unmethylated CpG oligodeoxynucleotide, an immunostimulatory agent, which served also as a carrier for doxorubicin [3] was conjugated with targeting moiety, an aptamer recognizing prostate-specific membrane antigen. This bioconjugate was found cytotoxic against human prostate cancer cell lines *in vitro*. Moreover, its tumor inhibitory effect was demonstrated using the mouse model.

Increased biotin receptors expression has been observed on various tumor cell lines. Therefore, biotinylation of carriers can be used to improve drug targeting into tumor cells. In order to improve the specificity of the dendrimer-mediated drug delivery into tumor cells, PAMAM dendrimers loaded with cisplatin were prepared. Their cytotoxic capacity against ovarian cancer cell lines has been recently demonstrated [67].

Another PAMAM dendrimers have been investigated for their capacity to cross human placenta [40]. The results of this study suggest that drugs conjugated to dendrimers display only limited penetration across the human placenta when compared to small drug molecules alone. Those conjugates give opportunity to deliver drugs selectively to pregnant women under condition, in which the drug transfer to fetus is limited.

Oligo-branched peptides have been proved to bind tumor cells that can be effective either for overexpression of peptide receptors or to kill them when coupled to cytotoxic compounds. The proof of concept has been shown for tetra-branched peptides containing neurotensin (NT) sequence that selectively targeted agents for human colon, pancreas, and prostate cancer *in vitro* and *in vivo* [15]. Fluorophore-conjugated peptides were able to discriminate healthy tissue from the tumor mass in human surgical samples demonstrating that neurotensin receptors can be used as tumor biomarkers. Branched peptides were conjugated with cytotoxic drugs either as uncleavable adducts or drug-releasing molecules. It has been shown in experiments evaluating the cytotoxicity of particular conjugates *in vitro*, using human cell lines from colon (HT-29), pancreas (PANC-1), or prostate (PC-3) carcinoma, that branched NT conjugated with MTX and 5-FdU was the most active agent on PANC-1 (EC_{50} 4.4×10^{-7} M) and HT-29 (1.1×10^{-7} M) cells. In vivo treatment with tetra-branched NT conjugated with 5-FdU resulted in a 50 % reduction in tumor growth in HT-29-xenografted mice, as compared to animals treated with the free drug.

A family of amphiphilic block glycopolymers containing glucose, galactose, and mannose were prepared via metal-free organocatalyzed ring-opening polymerization of functional cyclic carbonates generating narrowly dispersed products of controlled molecular weight and end-group fidelity. Their capacity to deliver drugs was investigated [56]. Prepared glycopolymers self-assemble into micelles having a high density of sugar molecules in the shell, a size less than 100 nm, and narrow size distribution. They displayed little cytotoxicity, which is important for drug delivery. In experiment using galactose-containing micelles as an example, specific targetting of ASGP-R positive HepG2 liver cancer cells compared to ASGP-R negative HEK293 cells was observed, due to the ASGP-R-mediated endocytosis, although the galactose was attached to the carbonate monomer at 6-position. The enhanced DOX-loaded galactose-containing micelles by HepG2 cells significantly increased cytotoxicity of DOX as compared to HEK293. This new family of amphiphilic block glycopolymers has great potential as carriers for targeted drug delivery specifically to liver tissues/cells.

It has been shown that β-1,3-glucans, such as schizophyllan and curdlan, can form a complex with polynucleotides through hydrogen bonding between the two

main chain glucoses and the one nucleotide base. As reviewed [41], these structures can be used as carriers for polynucleotides with a therapeutical potential, such as antisense DNA, siRNA, and CpG ODN. Since β-1,3-glucans can be recognized by dectin receptors, present on antigen presenting cells including dendritic cells and macrophages, their carrier capacity could be used to modulate antigen presentation and priming of immune responses.

An increase of anti-toxoplasmic activity up to 7 orders of magnitude was achieved by complex of sulfadiazine with PAMAM dendrimer [10, 48].

The guanidinium groups in dendrimer shell play a crucial role for facilitation of the transport of dendritic polymers through liposomal and cell membranes [60].

Hyperbranched polyamidoamines (HPMA) with β-CD were used for controlled drug release of chlorambucil [69]—an anticancer drug. Chlorambucil complexation increased glass transition temperatures of hyperbranched polymer. The higher content of β-CD, the slower release of chlorambucil.

G4-lysine MAPs with PEG-1000 core and galactose coating were studied for drug delivery of chloroquine phosphate [1]. In contrast to uncoated dendrimer, galactose coating reduced almost 5 times phagocytosis and dramatically the hemolytic toxicity. Generally, the coating led to much more safe controlled delivery of chloroquine than the uncoated formulations.

Electron paramagnetic resonance (EPR) spectroscopy is a useful tool for studies of entrapment and release of drug from dendrimeric carriers [36]. EPR analysis of the guest–host behavior is based on detection of paramagnetic probes—nitroxides. EPR can reveal dependence of drug entrapment and release on the dendrimer generation and on their interactions with molecular species.

Overexpressed fibroblast growth factor receptor (FGFR) serves as an important target for drug delivery [61]. The conjugate of FGF-1 with G5-PAMAM was transported to nucleus and cytosol; therefore, these conjugates can be a platform for drug delivery to specific tumor cell compartments and as an FGF delivery agent for the angiogenesis and wound healing.

A dendrimeric nanocluster with folate and a fluorescent dye attached at different ends is held together by DNA. The nanocluster specifically targets cancer cells and interacts with folate receptors on the cell surface [12].

Inhibition of mitosis was achieved by glycopeptide dendrimer conjugates of colchicine. These MAGs with 4 or 8 glycoside units on their surface (β-Glc, α-Gal, α-GalNAc, or Lac) facilitated drug delivery of colchicine. They are selective vehicles for the delivery of cytotoxic compounds to cancer cells [14, 29].

A role of dendrimer generation and PEG length in the biodistribution of PEGylated dendrimers was studied [28]. Diethylenetriaminepentaacetic acid (chelating agent) was attached to PEGylated PAMAM and labeled with radioactive indium. The conjugation increased time of drug in blood circulation and suppressed the accumulation in normal organs such as the kidneys and the liver.

A potential candidate for solid tumor treatment was prepared by huge PEGylation of PAMAM dendrimer-doxorubicin conjugates [70]. These conjugates were delivered into the tumor cells.

Nanoparticles from star PAMAM-b-poly(ε-caprolactone)-b-poly(gluconamidoehylmethacrylate) were studied as carriers of nimodipine and as biomolecular binders of Con A with the aim to develop a drug against hemorrhage [13].

The G2 and G3 of dendrimers with Asp oligopeptides in the periphery and naproxen in the core were prepared [44]. These systems bind tightly hydroxyapatite *in vitro* and allow the use of peptide dendrimers for bone targeting.

G4 PAMAM dendrimer increased the solubility of piroxicam 222 times and prolonged the piroxicam half-life of elimination [47].

A nanoscopic delivery system with a size of 5–10 nm was described [21]. The conjugated dendritic carrier with up to 25 copies of peptidic cargo promoted rapid cellular uptake by NIH 3T3 cells. This led to a new way for the design of protein and oligonucleotide drugs.

Specific delivery of drug to liver [5] and brain [35] was achieved.

Supramolecular pseudopolyrotaxanes mimicking strings with pearls were prepared from various CD (pearls) and linear polymers (string) such as poly(ethylene) glycol (PEG), poly(propylene)glycol, and poly(tetrahydropyran) [43]. Derivatization of the termini converts these pseudorotaxanes to rotaxanes, which are efficient devices for drug and gene delivery [32, 65].

β-CD with 17-β-estradiol served for selective delivery of the conjugate to cytoplasm and provided extranuclear estrogenic effect without side effect on genetic apparatus [27].

Bioreducible hyperbranched copolymer (H40-star-PLA-SS-PEG) with Boltorn® H40 core, poly(L-lactide) (PLA) inner shell, and PEG outer shell and disulfide linkages between the hydrophobic and hydrophilic parts was studied as a source of unimolecular micelles suitable for smart drug delivery triggered by reduction [45]. In oxidized form, unimolecular micelles in aqueous solution with an average diameter of 19 nm are formed by H40-star-PLA-SS-PEG. Under reducing conditions of 10 mM dithiothreitol (DTT), the micelles formed aggregates of large particles. Doxorubicin (a hydrophobic anticancer drug) was loaded into these micelles and easily released under reductive conditions. Loaded doxorubicin was slightly better than free drug in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Triggered smart delivery can be achieved by these bioreducible unimolecular micelles during cancer therapy.

A potential of three hyperbranched polymers, an unmodified pseudo-fourth generation dendritic polyester based on 2,2-bis-(methylol)-propionic acid with 64 terminal OH groups (Boltorn® H40), a lipophilic, fatty acid modified dendritic polymer (Boltorn® U3000), and an amphiphilic dendritic polymer (Boltorn® W3000) for delivery of paclitaxel has been investigated [49]. It has been shown that all three branched polymers form nanoparticles endowed with a potential to serve as delivery systems for paclitaxel. Due to their high loading efficiency, Boltorn® U3000 and W3000 represent the most interesting candidates.

Another hyperbranched polymer for smart delivery was prepared [19]. The polymer formed 60 nm micelles with a reversible thermosensitive phase transition at 32 °C. It can deliver a drug to the site with elevated temperature, where the micelles release their cargo.

Other examples of smart drugs or stimuli responsible systems were presented [33, 63].

Amphiphilic polypeptide dendrimers based on block polymer of (G3-glutamic acid)-b-polyphenylalanine form micelles with relatively low critical micellar concentration below 20 μg/mL [64]. They were used for delivery of doxorubicin to HepG2 liver cancer cells. The delivery provided death to 75 % of the cancer cells. The drug was released within 60 h.

The oral route is the most convenient route for drug administration. However, a number of drugs cannot effectively cross the intestinal epithelium into the blood circulation. PAMAM dendrimers are known to permeate across intestinal epithelial barriers so they have been suggested to be used as oral carriers for drugs with poor bioavailability after the oral administration. Cytostatic agent doxorubicin, a P-gp and CYP substrate, has been incorporated into PAMAM complex for oral administration and tested for bioavailability [26]. Caco-2 cell line was selected for testing since this cell line is known to express P-gp and CYP. The cellular uptake of doxorubicin in Caco-2 cells treated with the doxorubicin–PAMAM complex was increased significantly with an increase in concentration and time, as compared to that treated with free doxorubicin. Further, the transport efficiency of the doxorubicin–PAMAM complex from the mucosal side to the serosal side was 4–7 times higher than that of free doxorubicin in different segments of small intestines of rat. Collectively, the doxorubicin bioavailability, when complexed with PAMAM, was more than 200-fold higher than that of free doxorubicin after oral administration. These results indicate that PAMAM dendrimer can be efficiently used to deliver drugs across small intestine epithelial cell. The mode of action is probably suppression of drug efflux by P-glycoprotein and prevention of metabolism of the drug on cytochrome P-450.

Mannosylated solid lipid nanoparticles were used for site-specific delivery of doxorubicin to A549 tumor cells [24]. The nanoparticles were most toxic toward the tumor cells in comparison to neat drug and unloaded nanoparticles. Moreover, the loaded particles have shown almost no hemolysis and circumvented kidney and liver damage. Hence, they delivered the drug selectively and specifically to tumor tissue.

Different drug delivery systems in HIV pharmacotherapy, including different sorts of dendrimers, have been reviewed [55].

Conjugation of drugs to a dendrimer has been evaluated for improved retaining of drugs within the lung when treating inflammatory disorders, such as asthma. On mice model of asthma, the dendrimeric conjugate of G4 PAMAM with methylprednisolone was explored for pulmonary drug delivery [23]. Conjugation increased retaining of drugs in the lung during the asthma treatment. Dendrimers have been tested as nanoscale intracellular drug delivery vehicles designed for the improved bioavailability of methylprednisolone (MP), a corticosteroid used in the treatment (through inhalation) of lung inflammation associated with asthma. Methylprednisolone was conjugated to G4-PAMAM dendrimers and solubilized in lysine carrier and the capacity of conjugate to improve the airway delivery was

evaluated in a pulmonary inflammatory murine model that was based on an 11-fold enhancement of eosinophil lung accumulation following five daily inhalation exposures of sensitized mice to the experimental allergen ovalbumin. Five daily trans-nasal treatments with the carrier alone, free MP, and MP–dendrimer at $5\,mg\,kg^{-1}$ (on a drug basis) did not induce additional lung inflammation, although free MP decreased baseline phagocytic cell recoveries by airway lavage and tissue collagenase dispersion. MP treatments alone decreased ovalbumin-associated airway and tissue eosinophil recoveries by 71 % and 47 %, respectively. Equivalent daily MP dosing with MP–dendrimer conjugate further diminished these values with decreases of 87 % and 67 %, respectively. Thus, conjugation of MP with a dendrimer enhanced the ability of MP to decrease allergen-induced inflammation, perhaps by improving drug residence time in the lung since only 24 % of a single dose of dendrimer delivered to the peripheral lung is lost over a 3-day period. The conjugate was approximately 1.2–1.4 times more efficient in decrease of allergen-induced inflammation than the free MP.

Two radiodense elements were incorporated into one contrast agents [20]. This provide high-performance contrast agents for biomedical CT imaging applications.

The topics of dendrimer solubility (both dendrimers as such and their influence to drug solubility), drug delivery, dendrimer biocompatibility, and toxicity are closely related and influence each other [2, 4, 7, 17, 51, 53, 54, 57–59].

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Chapter 14
Dendrimers in Gene Delivery

Delivery of nucleic acids to target cells can be achieved by both viral and nonviral delivery systems [7,8,18,20,22,32]. Both of these methods have distinct advantages and disadvantages. In contrast to viral vectors, the nonviral ones provide the advantage of safety and flexibility. The disadvantage of nonviral carriers is their low efficiency. Inherent properties of dendritic nanoscale devices provide an ideal tool for development of synthetic (nonviral) carriers for delivery of nucleic acids [7]. Among these properties, the defined architecture, high ratio of surface groups to molecular volume, and difference of hydropathic properties of interior and exterior are the most important for drug and gene delivery.

Dendrimeric structures have been suggested to be used in gene therapy as potent carriers capable to, sometimes specifically, deliver DNA or RNA into the cells. Dendrimers are able to form complexes with many types of nucleic acids, such as plasmid DNA, antisense oligonucleotides, and RNA. Due to their defined architecture and a high ratio of multivalent surface moieties to molecular volume, they have become very important for the development of synthetic vectors for nucleic acid delivery [7]. Indeed, dendrimer-based transfection agents have become routine tools for many molecular and cell biologists, but the therapeutic delivery of nucleic acids complexed with dendrimer should be further investigated. The cationic dendrimers interact with the anionic backbone of nucleic acids mainly electrostatically. Complexed DNA is protected against degradation and the net positive charge of the dendrimer nucleic acid complex determines the transfection efficiency. However, highly cationic complexes are cytotoxic. Their properties can be tuned by many factors such as concentration of dendrimer amines and nucleic acid phosphates, salt concentration, stoichiometry, bulk solvent properties like pH, and buffer strength [7, 8]. For a development of dendriplex complexes with nucleic acids which can be used in gene therapy or, in general, as transfection reagents, knowledge of their transfection efficiencies and cytotoxicities is crucial. For transfection efficacy of reporter genes, standard techniques have been summarized [25] such as luciferase reporter assay, β-galactosidase assay, or green fluorescent protein microscopy. The cytotoxicity is usually determined by standard MTT test.

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The C_{60} fullerene (buckminsterfullerene or buckyball) is a stable compound consisting of a polygon with 60 vertices and 32 faces, 12 of which are pentagonal and 20 hexagonal. Due to its unique chemical and physical features, C_{60} have a potential to be used in many fields both in biological and material chemistry. C_{60} buckyballs are able to intercalate into biological membranes, destabilizing them. Chemical modification can break the spherical symmetry, creating two well-differentiated poles [18]. By modification with functional groups that are charged in water, such as carboxylate or amine groups, the extremely hydrophobic C_{60} can be made water soluble. These derivatives can interact with biomolecules and their ability to form aggregates with DNA qualifies them as potential carriers in gene therapy.

Dendrimer has also been utilized for RNA delivery, such as small interference RNA (siRNA). RNA interference (RNAi) is a widely used, routine, and powerful tool for selectively silencing gene expression which is widely used in basic research, and it has also therapeutic potential. Inefficient delivery of exogenous short interfering RNA (siRNA) molecules to cells and tissues represent one of the obstacles for therapeutic development. Delivery vectors must be designed to effectively complex with nucleic acid molecules as well as to facilitate overcoming intracellular barriers such as endosomal escape and cytoplasmic vector dissociation. Delivery of siRNA via different complexes, such as branched PEI or poly-D,L-lactide-co-glycolide (PLGA), was covered by an excellent review [31].

PAMAM nanoscopic, spherical polymers are under investigation as DNA carriers for their capacity for efficient gene encapsulation. A question was whether they could serve as a base for a complex carrier for brain-targeting gene delivery capable to penetrate through the blood–brain barrier (BBB) [12]. The 29 amino acid peptide conjugate derived from the rabies virus glycoprotein (RVG29) [The peptide RVG29 with a cysteine on *C*-terminal (YTIWMPENPRPGTPCDIFTNSRGKRASNGC) Ltd.], serving as a ligand for brain targeting was modified on PAMAM through bifunctional PEG which was then complexed with DNA, yielding PAMAM–PEG–RVG29/DNA nanoparticles (NPs). These NPs were uptaken by brain capillary endothelial cells through a clathrin- and caveolae-mediated energy-depending endocytosis which could be inhibited by free RVG29 and GABA but not by nicotinic acetylcholine receptor (nAChR) agonists/antagonists. This suggested that RVG29 probably relates to the GABAB receptor besides nAChR reported previously. PAMAM–PEG–RVG29/DNA NPs displayed higher BBB-crossing efficiency than PAMAM/DNA NPs in an *in vitro* BBB model. *In vivo* imaging showed that the NPs were preferably accumulated in brain and also the report gene expression of the PAMAM–PEG–RVG29/DNA NPs was observed in brain (significantly higher than unmodified NPs). The mechanisms of this efficient gene delivery vector are not fully understood and they are under investigation. Thus PAMAM–PEG–RVG29 represents the nonviral vector that can be efficient for noninvasive and brain-targeting gene delivery.

Due to electrostatic and hydrophobic interactions, dendrimers can form complexes with various forms of nucleic acids, such as plasmid DNA, antisense oligonucleotides, and RNA [22]. The complexation improves not only the solubility

of nucleic acids but also protects it from degradation. Nucleic acids are polyanions, which can be easily captured by polycationic dendrimers. The transfection efficiency depends on the net positive charge of the dendrimer nucleic acid complex. Unfortunately, high positive charge causes significant cytotoxicity. There are several factors influencing the final properties of nucleic acid–dendrimer complexes such as stoichiometry, concentration of dendrimer amines and nucleic acid phosphates, bulk solvent properties like pH, salt concentration, and buffer strength [7, 8].

The use of PAMAM dendrimers in gene delivery was reviewed [20, 21, 32]. Clinical applications of PAMAM dendrimers in gene delivery are limited due to their cytotoxicity, reticuloendothelial system uptake, and hemolysis [32].

Partial acetylation of amino groups decreased toxicity of G5 PAMAM dendrimers and facilitate delivery of siRNA vectors [28]. Up to 60 % acetylation yielded siRNA complexes with diameter around 200 nm. The high extent of acetylation was responsible for lowered toxicity and facilitated release of siRNA.

Development of new polymeric carriers is complicated by the fact that most chemical modifications affect multiple aspects of the delivery process. The effects of primary amine acetylation of G5 PAMAM dendrimers used as a vector for siRNA to U87 malignant glioma cells have been investigated [28]. Modified vectors have been prepared by PAMAM dendrimer reaction with acetic anhydride to obtain controlled extents of primary amine acetylation. It has been shown that acetylated dendrimers were complexed with siRNA and with up to 60 % of primary amines acetylated formed 200 nm complexes with siRNA. Importantly, increasing amine acetylation resulted in reduced polymer cytotoxicity to U87 cells as well as enhanced dissociation of dendrimer/siRNA complexes. However, acetylation of dendrimers reduced the cellular delivery of siRNA and confocal microscopy demonstrated that escape from endosomes was a major barrier to siRNA delivery in this system. It has been concluded that only partial acetylation (approximately 20 %) of PAMAM does not reduce the siRNA delivery efficiency of unmodified PAMAM.

PAMAM dendrimers containing pentaerythritol core (PEC) and 12 branches were prepared [29]. These G3–G5 PEC dendrimers condensed DNA into slightly charged nanoscale complexes. The PEC dendrimers had higher transfection efficiency and lower toxicity than the commercial nonviral gene carriers such as PEI, MAP, and PAMAM dendrimers with an ethylenediamine core (G5–G7). Hence, the PEC dendrimers can be used as nonviral gene vectors.

G3 PAMAM conjugate with α-cyclodextrin (α-CD) served as carrier of short hairpin RNA-expressing plasmid DNA (shpDNA) [27]. The conjugates prevented the enzymatic degradation of shpDNA by DNase I.

PAMAM-hyperbranched poly(amido amine) dendrimers (HPAMAM) were investigated as an effective agent for gene delivery [30]. Terminal amino groups of HPAMAM were modified with phenylalanine yielding 30, 45, and 60 Phe units. Complexation of these dendrimers with plasmid DNA was studied. The HPAMAM-Phe_{60} was the most efficient transfection agent from this series and it was more efficient than commercially available PEI.

The impact of shell groups substitution on the capability of PAMAM dendrimer to form dendriplexes with DNA and transfect cells was evaluated using HEK293 and human mesenchymal stem cells [23]. It has been demonstrated that G4 PAMAM displayed better transfection efficiency than G3, G5, G6 PAMAMs, as well as G4 PAMAMs with partially protected amino groups. For example, 25 % protection of the NH_2 groups significantly impaired and 50 % protection abolished the G4 PAMAM ability to transfect cells. The data revealed that increased hydrophobicity decreased the ability of dendrimers to transfect cells. G4 PAMAM with 50 % amino-protection was highly hydrophobic and formed micelles in solution. The micelles were able to deliver pGFP into cells, which was in correlation with the results of ethidium bromide intercalation assays, as well as with ANS fluorescence studies. The capacity of the G4 PAMAM dendrimer to deliver neurotrophin-encoding plasmid into mesenchymal stem cells was compared to that of lipofectamine 2000. Indeed, lipofectamine 2000 turned out to be a more efficient carrier (transfection efficiency 18.5 %) than G4 PAMAM dendrimer (1.2 %).

Polycationic phosphorous dendrimers, which form complexes with DNA, were effective transfection agents with low toxicity [19].

A host family based on bis-(guanidinium)-tetrakis-(β-CD) tetrapod was described as an efficient nonviral vector for transfection of siRNA and DNA to human embryonic lung fibroblasts [15].

The cellular uptake of G2–G3 MAP dendrigraft with different extents of PEGylation is carried out by caveolae-mediated process and macropinocytosis [9]. Moreover, these dendrimers are biodegradable and nonviral vectors.

Gene delivery systems based on polycationic amphiphilic cyclodextrins (paCDs) were studied [6]. A chemoselectivity of primary versus secondary hydroxyl groups on the CD torus was used for regioselective decoration of each rim with cationic and lipophilic units, respectively. Their highly symmetrical structure is a blend of both cationic lipids and cationic polymers, the two most used types of nonviral gene vectors. Due to the modular synthesis of monodisperse paCDs, structure–activity relationship studies are easily available.

A series of monodisperse multivalent polycationic β-CD "click clusters" have been synthesized and screened as therapeutic pDNA carriers [26]. These highly water soluble macromolecules condensed and protected pDNA like the viral delivery vehicles. β-CD with the longest dendron arms were the most effective delivery vehicles. A possible use of these systems for clinically viable drug delivery vehicles is suggested.

A use of artificial viruses based on micellar glycocluster nanoparticles was studied for gene delivery. Details of size-controlled gene coating with glycocluster nanoparticles were described [1–4, 14, 17].

Brush dendrimers with β-1,3-glucan scaffolds such as schizophyllan and curdlan bind polynucleotides via multiple hydrogen bonds and serve for delivery of antisense DNA, siRNA, and CpG ODN [16]. Since these β-1,3-glucans are recognized by dectin-1 receptor, the nucleic acid cargo can be selectively delivered to macrophages and dendritic cells.

Since G6 lysine dendrimers were efficient in transfection of DNA to various cell lines without cytotoxicity, the same system was used for delivery of siRNA [10]. The successful delivery led to knockdown of two important enzymes of gluconeogenesis, i.e., glyceraldehyde-3-phosphate dehydrogenase and phospho-enolpyruvate carboxykinase. The knockdown reduced glucose production in rat hepatoma H4IIEC3 cells. Furthermore, knockdown of organic cation transporter 1 suppressed the ability of metformin to inhibit gluconeogenesis in H4IIEC3 cells. The genes important for metformin action could be found by analysis of genome-wide loss-of-function in the system by the siRNA library (RNAi-based phenotypic screening).

In another study, previously reported lysine MAP with 128 amino groups has been utilized as an siRNA carrier used in combination with the weak-base amphiphilic peptide, Endo-Porter [10]. The efficacy of this carrier was demonstrated by the effective knockdown of glyceraldehyde-3-phosphate dehydrogenase in rat hepatoma H4IIEC3 cells with low cytotoxicity. Further, this system was used for the knockdown of PEPCK in the same cell line, the rate-limiting enzyme for gluconeogenesis. This knockdown led to a reduction in glucose production. In a second experiment, knockdown of organic cation transporter 1, which is thought to be the gene that regulates metformin action, has successfully diminished the ability of metformin to inhibit gluconeogenesis. This siRNA-based system can be further used for identification of various genes playing a role in metformin action regulations.

Dendrimer technology has also been utilized for experimental suicide gene therapy using a model of prostate cancer with double targeting to HSV-thymidine kinase (TK) and ganciclovir system [5]. G5 PAMAM dendrimer modified by folate, which ensures preferential uptake by tumor cells, and plasmids encoding HSV-TK and connexin43 genes (driven by prostate-specific membrane antigen (PSMA) promoter) was used. In combination with gemcitabine, folate G5 PAMAM delivered PSMA-TK-Cx43, decreased prostate cancer LNCaP cell proliferation, and induced apoptosis *in vitro*. Moreover, inhibited tumor growth in the LNCaP xenograft animal model. Connexin43, which can restore the gap junction of intercellular communication, was employed to enhance the "bystander effect" of the gene therapy.

Peptide dendrimers are considered to be attractive carriers for gene delivery because of their flexibility, good biocompatibility, and water solubility. It is likely that new strategies such as click chemistry will help to overcome current difficulties in the synthesis of functional preparations of higher generations of dendrimers. Arginine functionalized peptide dendrimer-based vectors ranging from G5 to G6 have been synthesized via click chemistry, and their possible use for gene transfection was investigated *in vitro* and *in vivo* [13]. These dendrimers were able to condense DNA to complexes with size around 180–250 nm. Complexed DNA was protected from nuclease cleavage. *In vitro*, functionalized peptide dendrimers displayed high transfection efficiency that was independent on serum presence. Dendrimer Arg G5 with molecular weight of 17 kDa demonstrated good transfection efficacy (sixfold higher compared to branched polyetherimide) in breast tumor

models, and its good biosafety record was demonstrated both *in vitro* and *in vivo*. On the other hand, Arg G6 dendrimer (46 kDa) was proved to be significantly cytotoxic.

Increased DNA binding and gene delivery efficiency of PAMAM dendrimers has been shown after dendrimer entrapping of gold nanoparticles [24]. These complexes effectively compacted pDNA and their higher transfection efficiency was explained by the fact that the presence of gold nanoparticles helped to preserve the three-dimensional spherical morphology of dendrimers, which was associated with more efficient interaction between dendrimers and DNA.

Poly(*N*-substituted acrylamide)s with various alkylamine side chain (from 2 to 12 methylene groups) were studied for delivery of plasmid DNA [11]. The brush dendrimers were less toxic than PEI control. The size of formed nanoparticles ranged from 100 to 350 nm. The most effective transfection efficiency was achieved for the polymer with 8 methylene groups in side chain. The uptake did not limit the transfection efficacy of this polymer.

The aforementioned examples clearly demonstrate the practical utility and potential of dendrimers in the field of gene delivery.

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Chapter 15
Dendrimers and Bacteria

Peptide, glyco, and glycopeptide dendrimers, capable of binding bacterial polysaccharides, represent attractive tools both for the therapy and for the diagnostics of the diseases of bacterial etiology. Finding new antibacterial drugs, as well as diagnostic tools, is nowadays of particular importance due to enhanced resistance of bacteria toward common antimicrobial agents. Indeed, the resistant microorganisms caused severe diseases with dramatic impact on mortality, disability, and economics. Attractive targets are bacterial lectins; some of them initiate infection by their interaction with the host cell glycoconjugates [49]. Free saccharides bind to the bacterial lectins with low affinity which is an obstacle for their therapeutic or diagnostic use. Notably, this can be overcome by their attachment to polymeric carriers or presentation as dendrimers resulting in strong multivalent binding. A critical overview about achievements in exploring the potential of dendrimers (PAMAM, PPI) as bactericides, as well as, an analysis of the factors influencing their biocidal activity was described [5].

15.1 *Escherichia coli*

Food contamination with *Escherichia coli* pathogenic strains results in frequent outbreaks of infections. Therefore, rapid and reliable detection of pathogenic bacteria is a key step in avoiding the spread of contamination. The carbohydrate-binding proteins on the bacteria surface are responsible for binding to host cells. Importantly, invasive strains of *E. coli* display high levels of mannose-binding proteins (lectins). These cell-surface molecules can represent targets both for novel diagnostic agents and for antibiotics. For example, *E. coli* and several other enterobacteria use type 1 fimbriae—30 kDa lectin-like subunit FimH—for mannose-mediated specific binding during anchoring to the host [6, 21, 29, 33, 43, 52]. Plant lectins, particularly Con A, *Dioclea grandiflora*, and pea lectins, bind mannopyranosides, e.g., high-mannose oligosaccharides [7, 8, 10, 15, 41, 43, 53]. Thus these lectins can interfere with the bacteria–cell interactions. A better understanding of the

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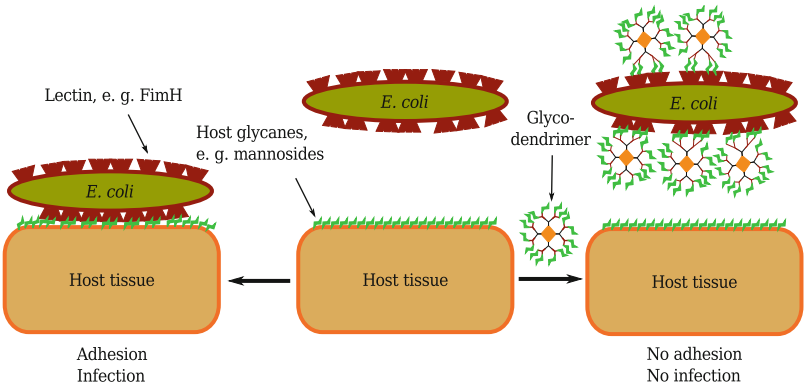

Fig. 15.1 Role of glycodendrimers in prevention of host colonization by bacteria [33, 45, 53]. The lectin–glycan interactions between bacteria and host start the adhesion process and thus infection *(left side)*. The infection is prevented by a saturation of bacterial lectin with suitable glycodendrimer (*right side* of the figure). The figure was reprinted from [45] with kind permission of Springer, Wien–NewYork

adhesion phenomena on a molecular basis is necessary in order to find a way to new antibiotics [7, 15, 33, 43, 53, 55]. Adhesion can be interfered by glycomimetics that can inhibit cellular recognition and the colonization of host tissues by pathogens (Fig. 15.1) [52]. Glycodendrimeric structures should increase the efficacy of the blocking through their multivalent effects leading to the enhancement of weak interactions of saccharides.

So far, the design and applications of glycodendrimeric mannosylated inhibitors of fimbriae caused adhesion were reviewed in several articles [7, 8, 15, 29, 43, 53]. Moreover, dendrimeric glycopeptide-based antibacterial vaccines have been developed (for a review, see [40]). Mannosylated glycodendrimers are under intensive investigation against gastrointestinal and urinary tract infections caused by *E. coli* [12]. It has been shown that mannosylation of spacered glucose and oligosaccharide (α,α-trehalose, β-melibiose, raffinose) cores provided carbohydrate-centered (octopus) mannosides [12]. The functional studies revealed that their relative inhibitory potency (RIP) of type 1 fimbriae-mediated adhesion was caused rather by a macromolecular than cluster effect. Therefore, the same authors prepared clustered glycomimetics with 12 α-mannose units as model compounds that enabled them to study multivalent interactions with glycocalyx constituents [52]. In this study of the anti-adhesive properties of type 1 fimbriated *E. coli*, RIP of this dendrimer—190—was ranked above methyl α-mannoside (RIP $=$ 1). In another study, nonavalent cluster mannosides have shown very poor inhibitory activities of *E. coli* type 1 fimbriae-mediated adhesion [29]. For better understanding of glycocalyx interactions, trivalent MAGs with mannose and L-fucose were anchored on golden surface via thiol-functionalized alkane and alkane-oligoethylene glycol spacers [18]. Syntheses of other glycocalyx mimetics were also described [48].

Another glycodendrimeric preparation has been developed and tested as potential drug candidate for *E. coli*-caused gastrointestinal and urinary tract infections

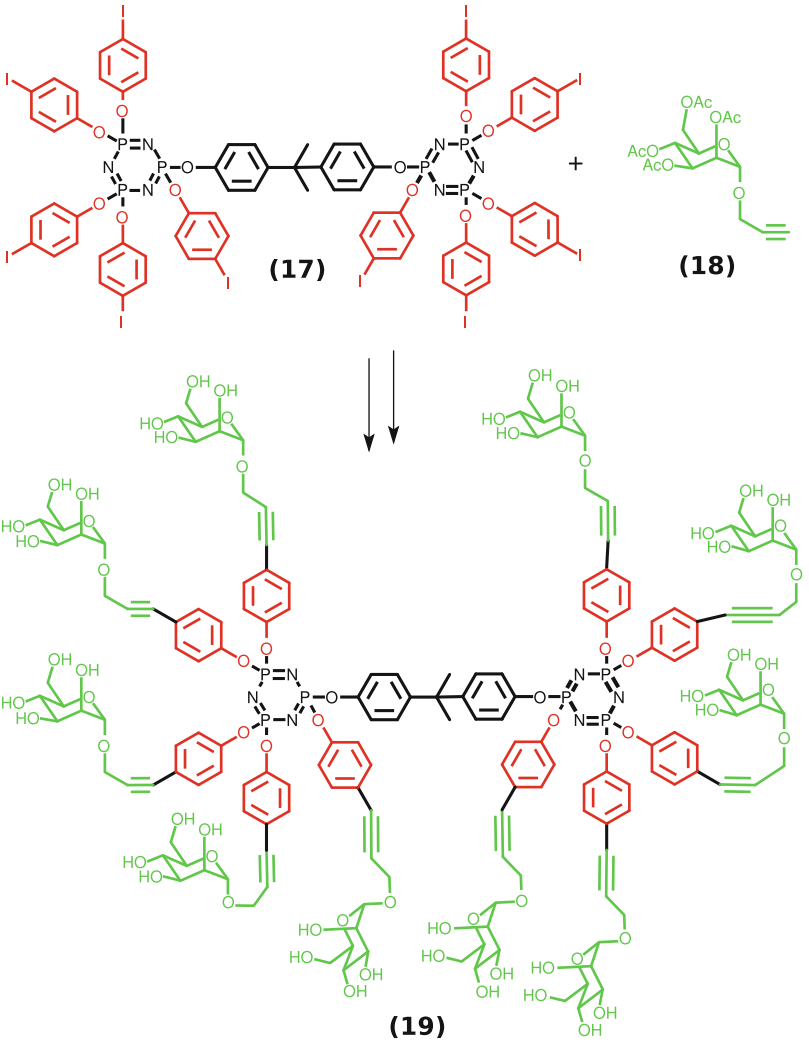

Fig. 15.2 Synthesis of "Majoral-type" multivalent glycodendrimers bearing covalently bound α-mannopyranosides onto a cyclotriphosphazene scaffold assembled using single-step Sonogashira coupling [54]. The figure was reprinted from [45] with kind permission of Springer, Wien–NewYork

[54]. They developed an efficient strategy for the first synthesis of "Majoral-type" [47] multivalent glycodendrimers with α-mannopyranosides covalently bound onto a cyclotriphosphazene scaffold assembled using single-step Sonogashira and 1,3-cycloaddition click chemistry (Fig. 15.2). The series of dendrimers with valencies 6–18 was tested by a well-established kinetic turbidimetric assay with Con A. The experiments revealed that the fastest and more complete formation of the insoluble cross-linked lattice was achieved with the decavalent dendrimer

(Fig. 15.2 structure **19**) containing alkyne spacer. Importantly, higher flexibilities of dodecamer and octadecamer were associated with lower cross-linking potencies compared to the decavalent dendrimer. The observed significant cross-linking enhancement was explained by more favorable extended intersugar distances, which facilitate the penetration into the carbohydrate's active site and permit a higher protein cross-linking ability [54]. It has been suggested that these α-mannopyranoside dendrimers can serve either as probes or effectors of biological processes involving multivalent carbohydrate-binding proteins.

Another strategy for a development of glycodendrimeric inhibitors of *E. coli* infection has been developed [14]. The methodology was based on iterative synthesis of spaced glycodendrons as oligomannoside mimetics with 3,6-diallylated carbohydrate as core molecule. Hydroboration, oxidation, and glycosylation with branched and unbranched sugar trichloroacetimidates were used for dendritic growth. Six of the synthesized glycodendrons were tested by ELISA as potential inhibitors of *E. coli* type 1 fimbriae-mediated bacterial adhesion. The data obtained were discussed with regard to spacer characteristics and sugar valency. It has been shown that all of the glycodendrons proved to be better inhibitors than MeMan. It has been also demonstrated that the thiahexyl-spacered glycodendrons were generally better inhibitors than the propyl-spacered ones, probably due to increased conformational flexibility and enhanced lipophilicity of the spacers. Further, oxidation of the sulfides into less lipophilic sulfones decreased the inhibitory potency of the corresponding compound by approximately one order of magnitude.

Dendrimer technology could be also used for sensitive detection of bacteria. Biocompatible glyco-dendronized poly-lysine polymers carry either three or nine mannose- or galactose-bearing dendrons that selectively bind, and thus can be used to detect bacteria. Central to the synthesis of glyco-dendronized polymers was the development of a continuous flow [2 + 2] photocycloaddition reaction to connect the dendrons and poly-lysine [22].

Glyco-dendronized polymers cluster bacteria by binding to cell-surface carbohydrate receptors [59]. This enables an easy read-out using microscopic analyses. Fluorescent hyperbranched poly(amidoamine) with mannose groups was proved to bind *E. coli* cell surface. Bacteria could be detected at concentrations higher than 10^2 cfu/mL.

Hexadecavalent dendrimers with mannose possessed the inhibition of hemagglutination titer of 1 μM [28]. This corresponds to relative inhibitory potency 500 times higher than that of the monovalent methyl α-mannopyranoside. These hexadecavalent dendrimers were prepared by powerful combination of diazo transfer reaction and click chemistry.

15.2 *Vibrio cholerae*

Glycodendrimeric preparations are also under investigation as anti-cholera drugs or as sensitive sensors for diagnostics [3, 4, 10, 23–25, 32, 34–37, 56, 58]. Cholera

toxin recognizes the cell membrane through specific interactions with ganglioside GM1 (Fig. 22.1). Galactose containing di-, tetra-, and octavalent dendrimers were prepared via click chemistry and tested for their capacity to inhibit cholera toxin binding. It has been shown that their inhibition of cholera toxin binding was as strong as in the case of the natural ganglioside GM1 oligosaccharide [4]. Further, click chemistry with dendritic scaffolds and extended arms was used for synthesis of multivalent GM1os and GM2os dendrimers [36]. These dendrimers displayed strong multivalent binding to cholera toxin B subunit, with an excellent value of at least 380,000-fold higher binding per sugar for octavalent GM1os dendrimer than that of monovalent GM1os derivatives. These results might be important for development of very sensitive sensor applications.

Cholera toxin produced by *Vibrio cholerae* has an AB5 architecture shared by the *E. coli* heat-labile enterotoxins. This pentameric B5 structure binds specifically to the ganglioside GM1 (Galβ(1→3)GalNAcβ(1→4)(Neu5Acα(2→3) Gal-β1-Glc-ceramide) on the surface of intestinal epithelial cells and mediates entry into the cell via endocytosis. The A subunit is consequently cleaved and initiates the catalytic events that result in host dehydration and diarrhea. The cholera toxin–GM1 interactions are mediated mainly by two terminal sugars of the GM1 pentasaccharide, galactose and sialic acid. Multivalent, glycopeptide inhibitors have been designed for the treatment of disease and pathogen infections [24]. Their binding can be optimized with general changes in glycopeptide architecture, composition, and charge. Control of glycopolypeptide backbone extension and ligand spacing has been shown to modulate the inhibition of the cholera toxin B subunit pentamer by these polymers. The role of backbone charge and linker length in modulating the inhibition event has been further investigated. Peptides of the repeating sequence AXPXG (where X stands for positive, neutral, or negative amino acid) containing propargyl glycine were designed and glycosylated with Gal via click chemistry. Their ability to inhibit the binding of the B5 subunit of cholera toxin was evaluated. Glycopeptides with a negatively charged backbone displayed improved inhibition of the binding event relative to the other glycopeptides. The inhibition was also affected by the length of the linker between the peptide and the saccharide ligand. These data demonstrate that, besides appropriate saccharide spacing and polypeptide chain extension, saccharide linker conformation and the systematic placement of charges on the polypeptide backbone are also important for glycopolypeptide-based multivalent inhibitors.

The topic of cholera vaccines was reviewed [10].

15.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a human opportunistic pathogen. Several virulence factors are produced by *P. aeruginosa*, such as lectins LecA and LecB [13]. LecB is L-fucose-specific lectin, which serves for tissue attachment and the creation of

biofilms [9,11,16,17,19,20,26,50]. The compounds preventing biofilm formation or suppressing an adhesion event strongly protect host organisms against a colonization by these pathogens. The colonization is responsible for infections of the lungs, the urinary tract, and kidneys.

A library strategy provided powerful inhibitors of biofilm formation of *P. aeruginosa* [11, 16, 17, 19, 20]. A 15,625-membered dendrimeric library [19] with α-*C*-L-fucosyl residues at the *N*-termini was tested for binding to L-fucose-specific lectins. Selected glycopeptide dendrimer (L-Fuc-α-CH_2-CO-Lys-Pro-Leu)$_4$-(Lys-Phe-Lys-Ile)$_2$-Lys-His-Ile-NH_2 was a potent inhibitor of *Ulex europaeus* lectin UEA-I (IC_{50} 11 μM) and *P. aeruginosa* lectin PA-IIL (IC_{50} 0.14 μM) binding. In the next step, bigger library (390,625 members) of glycopeptide dendrimers provided compounds strongly disrupting established biofilms of the wild-type strain of *P. aeruginosa* and several clinical isolates [16, 17]. Further optimization of the structure activity relationships led to inhibitors of *P. aeruginosa* biofilm formation (L-Fuc-α-p-O-C_6H_4-CO-Lys-Ala-Asp)$_4$-(Lys-Ser-Gly-Ala)$_2$-Lys-His-Ile-NH_2 [16] and (L-Fuc-α-CH_2-CO-Lys-Pro)$_8$-(Lys-Leu-Phe)$_4$-(Lys-Lys-Ile)$_2$-Lys-His-Ile-NH_2 [20] with IC_{50} 0.11 and 0.025 μM, respectively. The most powerful inhibitor was 440 times more potent than the corresponding monosaccharide L-fucose [20]. The fucosylated glycopeptide dendrimers are polyvalent inhibitors of *P. aeruginosa* adhesion and biofilm formation (Fig. 8.1).

The adhesion of bacteria to human glycoconjugates can be inhibited by soluble glycomimetics that compete with the natural target. Four monovalent and one divalent α-L-fucosyl amides have been tested for their affinity for a L-fucose-binding lectin from *P. aeruginosa* [2]. It has been shown by isothermal calorimetric titrations that all preparations interact with the lectin in the micromolar range; however, the highest affinity was observed for the divalent ligand. Molecular modeling established that, compared to *O*-L-fucoside compounds, the glycomimetic amide group resulted in the loss of water-bridged hydrogen bonds that could be partially compensated by additional contact of the aglycone with the protein surface. Notably, α-*N*-L-fucosyl amides do not correspond to a known biological chemotype, and therefore, like *C*-glycosides, they ought to be stable to the action of enzymes which could be beneficial in a development of effective inhibitors of bacteria binding to bacteria to human glycoconjugates.

15.4 *Haemophilus influenzae*

Haemophilus influenzae type b (Hib) belongs to important human pathogens and was widespread in developed countries until application of successful conjugate vaccines in the 1990s [38]. Nevertheless, approximately 600,000 infant deaths are caused by Hib-induced pneumonia or meningitis in developing countries per year [30]. Wide use of the polysaccharides as vaccines serves for protection of adults and older children [1, 31, 39] and provides long-lasting immunity especially in infants

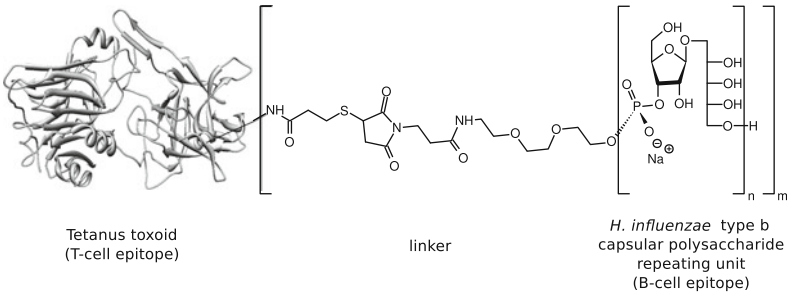

Fig. 15.3 Pioneering example of a commercial semisynthetic carbohydrate-based vaccine. Adapted from [42, 57]

[44]. Conjugation of polysaccharide with proteins provided globular multivalent structures—potent vaccines. Thus, the synthetic capsular polysaccharide antigen of Hib was used for preparation of the first commercially available semisynthetic vaccine [42, 57] (Fig. 15.3), which was tested in Cuba and has shown long protectivity against Hib. This opened a way toward many following vaccines based on similar principles [42].

15.5 *Mycobacterium tuberculosis*

The capability of intracellular pathogens (*Mycobacterium tuberculosis* and *Leishmania*) to hijack immune cells made the preparation of effective therapies challenging. Serrano-Gomez et al. and Song et al. [46,51] have shown that changes of surface oligosaccharides on artificial probes were sufficient to block IL-12 production by macrophages and thus dampen innate immune responses.

It is known that pathogen glycolipids, such as *Leishmania* spp. lipophosphoglycan and *Mycobacterium tuberculosis* mannosylated lipoarabinomannan, modulate interactions with host phagocytic cells. Recently, pathogen surface oligosaccharides have been synthesized and their modulation of the immune response has been investigated [27]. Trimannose cap carbohydrates from the mannosylated lipoarabinomannan and lipophosphoglycan altered the production of proinflammatory cytokines via a toll-like receptor-mediated mechanism. Trimannose treatment increased the production of other Th1 proinflammatory cytokines, such as IFN-γ, IL-6, and TNF-α, and *in vivo* treatment with trimannose led to increased Th1-polarizing, IL-12-producing cells from the draining lymph nodes of treated *Leishmania major*-infected mice compared with cells from untreated infected mice. These results might be of importance for a development of adjuvant therapy of chronic infections.

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Chapter 16
Dendrimers and Viruses

Dendrimers, as hyperbranched synthetic well-defined molecules, have been suggested as potential antivirals from several reasons, such as their small sizes (nanometers) and their ease of preparation and functionalization. Importantly they can be potent inhibitors of virus–cell interactions due to their multivalency and capacity to display multiple copies of surface groups (cluster effect) required for processes such as initial virus–cell interactions.

16.1 Influenza

Influenza represents a major health problem, as the pathogenicity of influenza virus is very high [12, 32, 42, 46] and there is still a lack of an effective drug treatment. Almost a quarter of million patients with flu are hospitalized every year in the USA. Death toll of the most severe "Spanish" flu was ca 20×10^6 people in 1918–1919 [32, 42]. Two unique glycoproteins, hemagglutinin (HA) and neuraminidase (NA), are localized on the surface of influenza A viruses. These glycoproteins play a decisive role in infection and replication and represent a potential target for therapy. HA is responsible for the binding of the virus to the cell that is being infected. Therefore, inhibition of the HA interaction with cellular receptors represents a widely studied strategy to block infection of influenza virus to host cells. HAs of influenza A viruses bind to its specific receptor—sialyl lactose (Neu5Ac-α-(2→3)-Gal-β-(1→4)-Glc), thus initiating infection through adhesion of influenza viruses to the surface of the host cell [17, 32, 42]. Dendrimers containing sialic acid as synthetic inhibitors of influenza virus have already been intensively studied [4, 21, 31, 37, 40, 41, 48, 60].

Furthermore, glycodendrimers may also possess the capacity to inhibit influenza neuraminidase. Recently, a set of influenza neuraminidase (sialidase) inhibitors have been synthesized and their effects were investigated [41, 42]. Twelve types of sialylated carbosilane dendrimers in a library containing thioglycosidic linkage, which is resistant to hydrolysis by the sialidases, were prepared. Dendrimers with

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3-, 4-, 6-, and 12 sialyl residues, and different spacer patterns, i.e., aliphatic linkage and ether and amide linkages, were tested: all of the ether- and amide-elongated compounds had inhibitory potencies for the influenza sialidases in the mM range.

In order to find more effective inhibitors, another series of carbosilane dendrimers functionalized with Neu5Ac-α-(2→3)-Gal-β-(1→4)-Glc moieties have been synthesized and tested for anti-influenza virus activity [32]. It was shown that their biological activities depended on the form of their core frame. The dumbbell(1)6-amide type glycodendrimer exhibited particularly high inhibitory activities against human influenza viruses [A/PR/8/34 (H1N1) and A/Aichi/2/68 (H3N2)] and displayed remarkably improved inhibitory activities in the μM level. These glycodendrimers are considered to be promising therapeutic agents for influenza disease.

HA is also a potential target for vaccines. As an example, experimental peptide-based vaccine against influenza virus inducing specific T-cell-mediated immune responses, important for reducing viral spread and accelerating the recovery from influenza, has been targeted against major surface glycoprotein HA. In this vaccine four T helper (T1, T3, T7, and T8) and four cytotoxic T lymphocyte (T2, T4, T5, and T6) epitopes have been coupled two copies of each to an orthogonally protected carrier SOC4—Ac-(Lys-Aib-Gly)$_4$–NH_2. These compounds were synthesized using combined SPPS and chemoselective ligation. They resembled the initial topology of the epitopes, crucial for their immunogenicity. Experiments in a murine model revealed that SOC4-conjugates comprising two copies of T7, T4, and T5 epitopes induced protective immunity and thus represent potential vaccine candidates [46].

16.2 HIV

Dendrimeric preparations have been investigated in HIV research for their ability to block virus–cell interactions, to inhibit virion assembly, as well as adjuvants to chemotherapy. HIV envelope glycoproteins are glycosylated and their carbohydrate moieties play an important role in the virus–cell interactions [3, 9, 11, 13–15, 17–20, 24–27, 29, 31, 33, 35, 38, 43, 45, 47, 53, 54, 56, 57].

Dendritic cells (DC) play an important role in HIV-1 infection. Through a mechanism named trans-infection, they can transfer the virus to T lymphocytes where viral replication occurs. Thus abrogation of the HIV transmission in the DC–lymphocyte synapse is a potential therapeutic target. HIV–DC interaction is mediated by the glycans of viral surface glycoprotein gp120 and the C-type lectin DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3–grabbing nonintegrin) expressed on DCs. Binding to DC-SIGN can promote HIV, as well as hepatitis C virus infection of T cells from dendritic cells. Thus inhibition of the gp120-DC-SIGN interactions represents an alternative strategy to block HIV infection [5, 25, 26, 43, 44, 49, 52, 57–59].

Tetrameric DC-SIGN recognizes *N*-linked high-mannose oligosaccharides ($Man_9GlcNAc_2$) through multivalent and Ca(II)-dependent protein–carbohydrate interactions. Carbohydrate-based antiviral agents mimicking the cluster presentation of oligomannosides on the virus surface were designed and tested [26]. Multivalent gold nanoparticle (manno-GNPs) library containing truncated $Man_9GlcNAc_2$ was prepared and screened for inhibitors of DC-SIGN binding to gp120. Moreover, these DC-SIGN ligands were also capable to interfere with the early steps of other infections via specific recognition of associated glycans. Different thiol containing spacers serving for attachment of the (oligo)mannosides to the gold surface were prepared. SPR was used to study their inhibition potency toward DC-SIGN binding to gp120. Complete inhibition of the binding in the micro- to the nanomolar range was achieved by the tested manno-GNPs. Millimolar concentration was required by the corresponding monovalent mannosides. Manno-GNPs containing the disaccharide α-Man*p*(1→2)αMan*p* were the best inhibitors of gp120 binding to DC-SIGN. Their activity was approximately 20,000-folds higher, as compared to the corresponding monomeric disaccharide.

Further studies showed that GNPs displaying multiple copies of the disaccharide Manα(1→2)Manα blocked DC-SIGN/gp120 binding at 120 nm in surface plasmon resonance (SPR) experiments [25]. Manno-GNPs coated with the linear tetrasaccharide Manα(1→2)Manα(1→2)Manα(1→3)Manα inhibited HIV trans-infection of human T cells similarly to GNPs coated with more complex branched penta- and heptaoligomannosides.

Another approach to inhibit DC-SIGN-mediated HIV infection has been used [43]. A new tetravalent dendron containing four copies of a linear trimannoside mimic was synthesized and its capacity to inhibit the trans-HIV infection process of CD4+ T lymphocytes at low micromolar range was demonstrated. The structural simplicity of trimannoside mimic on a Boltorn-type dendron enables large-scale production of highly water-soluble and noncytotoxic conjugate. The conjugate is stable at pH 5 for more than 1 week, which allowed topical vaginal application. unfortunately, at pH 7.4, the stability is reduced to less than half day. The anti-infective activity of the compounds was based on both the good affinity for DC-SIGN and on the tetravalent presentation on the dendron. Interestingly, the HIV antiviral activity is not influenced by viral tropism (X4 or R5) and this compound could serve as microbicide in a gel for topical application.

Another strategy in dendrimeric preparation usage against HIV was employed when 24 valent MAG containing sulfated cellobiose on the surface was investigated. It has been shown that branched, but not non-branched sulfated oligosaccharides blocked HIV-1 replication due to electrostatic interactions between negatively charged sulfated groups and positively charged gp120 [14]. The cluster effect was important for the biological activity since non-branched sulfated oligosaccharides have low anti-HIV-1 activity.

Multivalent approach was employed for a development of an alternative anti-HIV strategy based on the inhibition of HIV infection with the preparations of globotriose and 3'-sialyllactose. These carbohydrate head groups found on two glycospingolipids were covalently attached to a dendrimer core. These dendrimers

mimic specific glycosphingolipids present on immune cells that can be recognized as alternative receptors binding HIV-1 external envelope glycoprotein. They were capable to inhibit HIV-1 infection of T-cell lines and primary peripheral blood mononuclear cells by T-cell line-adapted viruses or primary isolates, with IC_{50} ranging from 0.1 to 7.4 μg/ml. These results suggest that the dendrimeric structures comprising specific glycosphingolipids could represent a novel class of HIV inhibitors [3]. Further, water-soluble hyperbranched β-galactosylceramide containing dendritic polymers bind HIV-1 gp120 and mimic multivalent glycosphingolipid display on cell surface (specific lipid rafts) required for assembly of the HIV-1 entry complex [29].

In another study, a series of catanionic multivalent analogues of β-galactosylceramide were synthesized and investigated [35]. Dendrimers based on cyclotriphosphazene core with phosphonic acid in the branches formed noncovalent complexes with *N*-hexadecylamino lactitol moieties. These supramolecular systems showed anti-HIV-1 activity. They displayed submicromolar IC_{50} in a cell-based HIV-infection model but also a high general cytotoxicity.

Another possibility is that dendrimers can be developed as anti-HIV-1 therapeutic agents targeting capsid assembly [9]. First generation of gallic acid triethylene glycol dendrimers harboring a large hydrophobic core and containing Lac shell were able to bind HIV capsid proteins and one of the tested dendrimers hampered *in vitro* formation of the HIV capsid.

Dendrimeric preparations are, together with other systems, under development as drug delivery systems in HIV pharmacotherapy [47]. An important task in delivery of anti-HIV/AIDS drugs is multifunctionalization of the nanocarrier system, such as, e.g., nanoparticles or dendrimers that could enable to incorporate several therapeutic agents in one carrier for maximum clinical effect [13]. PPI dendrimer or mannosylated fifth generation PPI increased 3TC antiretroviral activity against HIV-1. This approach is focused on targeting reservoirs such as quiescent CD4+ cells, mononuclear phagocytic cells (macrophages, dendritic cells, etc.), CNS, and male genital tract compartments [13].

The topic of dendrimeric glycopeptide antiviral vaccines was thoroughly reviewed elsewhere [11, 31, 39].

16.3 Other Viruses

A number of other viral infections have been suggested as targets of the dendrimer-based therapy [1, 6–8, 10, 16, 22, 23, 28, 30, 34, 36, 50, 51, 55]. Human papilloma virus (HPV) infections are the most common sexually transmitted infections (STIs). Importantly, infection with high-risk HPV types (primarily types 16, 18, 31, and 45) can cause cervical cell abnormalities that are precursors to cancer. Although two vaccines against most widespread HPV types are already on the market, there is still demand for antiviral treatments covering a broad spectrum of the HPV types. Topical antiviral microbicides that can block the full spectrum of genital HPV infections at the portal of entry could be used as complements to the vaccination.

Peptide dendrimers have been suggested as such potential antivirals. They consist of a peptidyl branching core and/or covalently attached surface functional units. Recently, a minilibrary of linear, dimeric, and dendrimeric peptides containing clusters of basic amino acids was evaluated for *in vitro* activity against HPVs. The dendrimer SB105-A10 was found to be a potent inhibitor of genital HPV types (i.e., types 16, 18, and 6) in pseudovirus-based neutralization assays with no evidence of cytotoxicity when used at 50 % inhibitory concentration (between 2.8 and 4.2 μg/ml, i.e., 0.59 and 0.88 μM, respectively) [10].

Classical swine fever virus (CSFV) affecting domestic and wild pigs is considered to be one of the most devastating diseases for the pig industry [28, 51]. Protection against CSFV is associated with the induction of neutralizing antibodies, although there is mounting evidence that T-cell responses play a role in protection against CSFV. Live attenuated vaccines against CSFV inducing high titers of antibodies have been used for a long time. However, especially because of antibody patterns in vaccinated animals are very similar to those observed in naturally infected animals, there is a demand for novel vaccination strategies, including those based on peptide vaccines. First generation of dendrimeric B4T-type CSFV vaccine candidates comprising B- and T-cell epitopes derived from the viral glycoprotein E2 has been constructed and construct $[KEDFRYAISSTNEIGLLGAO]_4K_2KKKHKVRNEVMVHWFGD$ (where O represents 3,6-dioxaoctanoic acid) can be considered as a promising CSFV vaccine candidate. Its partial protective effect was fully consistent with the neutralizing antibody titers elicited by the two protected animals and coherent with the induction of antipeptide antibodies and of IFN-γ-producing cells even in the absence of neutralizing antibodies before challenge [28].

Dendrimeric preparations have also been investigated as carriers of antiviral drugs, such as acyclovir [2]. A poly(amidoamine) brush dendrimer (PAA) with β-cyclodextrin was synthesized by polyaddition reaction of 6-deoxy-6-amino-β-cyclodextrin (β-CDNH2) and 2-methylpiperazine to 2,2-bis(acrylamido)acetic acid in aqueous medium. This β-CD/PAA copolymer bearing β-CD units along the macromolecular chain was water-soluble and noncytotoxic. This preparation was complexed with acyclovir, a poorly water-soluble drug. β-CD/PAA was able to solubilize up to 11 % w/w of acyclovir and substantially increased the aqueous solubility of the drug. The acyclovir β-CD/PAA complex exhibited a higher antiviral activity than the free drug.

One subclass of dendrimers are peptides on tetravalent MAPs. The ability of two peptide dendrimers to directly and almost completely inhibit human cytomegalovirus (HCMV) replication in both primary fibroblasts and endothelial cells was demonstrated [22]. This inhibition was specific for CMV as the agents were also found to inhibit murine CMV replication, whereas they were not able to inhibit adenovirus or vesicular stomatitis virus. The peptide dendrimers prevented adsorption of the HCMV to cells at 4 °C, whereas a dendrimer with a different amino acid sequence within the functional group and minimal anticytomegaloviral activity, was ineffective. The dendrimers bound to human cells through an interaction with cell surface heparan sulfate and thereby blocked virion attachment to target cells.

These type of compounds may be attractive candidates for a new class of drugs for targeting virus attachment. Their advantages are a lack of an induction of drug resistance. However, their antiviral capacity has not been studied *in vivo* in animal models of acute infection.

Herpes simplex viruses (HSV) are responsible for a wide variety of clinical manifestations. They range from asymptomatic infection or mild mucocutaneous lesions on the lips, cornea, genitals, or skin, up to more severe, and even life-threatening infections, including encephalitis, neonatal infections, and progressive or visceral disease in the immunocompromised hosts. Although HSV infections are often subclinical, their incidence and severity have increased over the past decades due to the increasing number of immunocompromised patients, including HIV-positive individuals. Dendrimer-based molecules are under investigation as potential antiviral agents. The ability of MAPs to directly inhibit herpes simplex virus 1 (HSV-1) and HSV-2 *in vitro* replication has been shown [23]. It has been shown that both $[ASLRVRIKKQ]_4K_2K$-β-Ala and its derivative $[ASLRVRIKK]_4K_2K$-β-Ala prevented HSV-1 and HSV-2 attachment to target cells, whereas $[NKKIRVRL]_4K_2K$-β-Ala, a dendrimer with a different amino acid sequence within the functional group and minimal antiviral activity, was ineffective in blocking HSV attachment. Importantly, this capacity was retained even under conditions mimicking those in vagina, a potential therapeutic location. Both $[ASLRVRIKKQ]_4K_2K$-β-Ala and $[ASLRVRIKK]_4K_2K$-β-Ala retained their ability to inhibit HSV adsorption at pH 3.0 and 4.0 and in the presence of 10 % human serum proteins, a potential therapeutic location for such compounds. The inhibition of HSV adsorption is likely associated with the ability of $[ASLRVRIKK]_4K_2K$-β-Ala to bind to the glycosaminoglycan moiety of cell surface heparan sulfate proteoglycans, thereby blocking virion attachment to target cells. Combination with acyclovir in checker board experiments $[ASLRVRIKK]_4K_2K$-β-Ala exhibited synergistic activity. Taken together, these findings suggest that $[ASLRVRIKKQ]_4K_2K$-β-Ala and $[ASLRVRIKK]_4K_2K$-β-Ala have a potential as topical microbicides for the prevention of HSV infections.

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Chapter 17
Dendrimers and Parasites

Dendrimeric preparations have also been studied as antiparasitic agents, either vaccines or therapeutics that would be able to cope with major health problems, such as with schistosomiasis or malaria. A promising strategy is based on the development of synthetic MAP-derived vaccines (reviewed by Tam and Niederhafner et al. [12, 19]) which enable to deliver multiple T-cell and B-cell epitopes as the constituents of a single immunogen. Schistosoma, a parasitic disease caused by five species of schistosome platyhelminth worms adapted to man, is present in endemic areas, which cover 74 countries in Africa, the Middle East, South America, and Southeast Asia where more than 600 million people are at risk and about 200 million are actually infected [1]. MAPs with different antigens, mainly derived from the primary sequence of *Schistosoma mansoni* glyceraldehyde 3-phosphate dehydrogenase (SG3PDH), have been tested for more than 20 years [2, 15]. It has been shown that MAPs with distinct combinations of SG3PDH-derived peptides display different efficiency against worm burden or egg count per worm [18]. Recently, immunogenicity of *S. mansoni ex vivo* lung-stage larvae excretory–secretory antigens and, moreover, MAPs derived from these proteins has been demonstrated [4, 5].

Novel therapeutic strategies are also necessary against malaria, treatment of which is complicated by several factors, such as drug resistance, the fact that *Plasmodium vivax* and *Plasmodium falciparum*, as the two species, distinct phylogenetically and antigenically, are responsible for the epidemics, or the necessity to target different developmental stages of the parasite [9]. Synthetic MAP vaccines tested in animals and also humans are usually derived from *P. falciparum* circumsporozoite protein [3, 8, 10, 20–22]. Recently, MAP vaccines harboring conserved protective epitopes from *Plasmodium* antigens from the sporozoite, liver, and blood stages of the life cycle that are widely recognized by populations of divergent HLA have been tested on several mouse strains [11] with results suggesting further pre- and clinical testing of these new-generation vaccines.

Another branched peptide has been designed for immunodiagnosis of *Fasciola gigantica*, an etiological agent of sheep fasciolosis, as well as for protection against

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this disease [7]. Several complexes of the peptide FasAc14p derived from a critical enzymatic site of cathepsin L proteinase, one of the defined antigens of *F. gigantica*, conjugated to sequential oligopeptide carrier Ac-[Lys-Aib-Gly]$_4$-OH have been studied and their capability to induce immune responses documented.

Dendrimeric structures have also a potential as antiparasitic drug carriers. An interesting theoretical molecular modeling study aiming to suggest a dendrimeric prodrug optimized in terms of its disassembly characteristics has recently been published [6]. A dendrimer consisting of myo-inositol core, L-malic acid spacer, and active agents such as 3-hydroxyflavone, quercetin, and hydroxymethylnitrofurazone has been investigated for the most likely enzymatic disassembly mechanism in order to find out potential antichagasic and antileishmanial prodrugs.

An incorporation of *Leishmania* into immune cells poses a challenge for the preparation of effective therapies. Serrano-Gomez et al. and Song et al. [16, 17] have proven that a modification of surface oligosaccharides on artificial probes was sufficient to block IL-12 synthesis by macrophages and thus dampen innate immune responses.

Lipophosphoglycan of *Leishmania* spp. modulates interactions with host phagocytic cells. Recently, *Leishmania* surface oligosaccharides have been prepared and their modulation of the immune response has been studied [13]. The experiments revealed that trimannose cap carbohydrates from lipophosphoglycan altered the production of proinflammatory cytokines. *In vivo* treatment with trimannose increased Th1-polarizing, IL-12p40 producing cells from the draining lymph nodes of treated *Leishmania major* infected mice, as compared to control untreated infected mice. More Th1 proinflammatory cytokines (IFN-γ, IL-6, and TNF-α) were also elevated. The results are significant for a development of adjuvant therapy of chronic infections.

PAMAM dendrimers can serve for early diagnosis of diseases related to *Trypanosoma cruzi* (Chagas' disease), *L.* spp., etc. [14].

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Chapter 18
Cancer

Dendrimers and dendrimer-based therapeutics provide promising field for development of new anticancer therapeutic strategies, as well as cancer diagnostics. They can be used as transporters of either encapsulated or conjugated antitumor agents that can be specifically delivered to the tumor via enhanced permeability and retention effect of the nanoparticle. Alternatively, they can target tumor cells specifically upon their conjugation with different agents such as antibodies, peptides, vitamins, or hormones. Dendrimers can be also used for diagnostic purposes as imaging agents. The crucial parameters for their utilization in nanomedicine are their long-term viability and biocompatibility. Indeed, novel dendrimers with biocompatible components and the surface modification of commercially available dendrimers by PEGylation, acetylation, glycosylation, and amino acid functionalization solved the safety problem of dendrimer-based nanotherapeutics [6]. (Glyco)dendrimers have also been investigated as antitumor drugs either as agents facilitating and enhancing tumor-antigen presentation by dendritic cells (DC). This improves the efficacy of antitumor vaccines. A major field in which the multivalent dendrimeric structures can take place is vaccine development. Indeed, aberrant glycosylation is strongly associated with cancer. Modification of the physicochemical properties of glycoproteins has a deep impact on their function and immunogenicity [1, 7, 11]. This topic has been reviewed [19]. Glycopeptide dendrimers containing different sorts of tumor-associated carbohydrate antigens (TACAs) (T_N, TF, sialyl-T_N, sialyl-TF, etc.; Fig. 22.1) were used in diagnosis and therapy of different sorts of cancer (lung, prostate, colon, etc.). These dendrimeric structures with incorporated T-cell epitopes and adjuvants can be used as antitumor vaccines. Best results were obtained with multiantigenic vaccines containing, e.g., five or six different tumor-associated antigens. The current state of the art in the field of dendrimer utilization as antitumor vaccines targeting TACAs is discussed in Chap. 22.

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18.1 Adjuvant Effects on Dendritic Cells

DCs are professional antigen-presenting cells with a unique ability to display tumor epitopes in the context of major histocompatibility complex class I and thus induce cytotoxic CD8+ T-lymphocyte-mediated immune responses. The mannose receptor and DC-SIGN, membrane lectins located on the surface of DCs, recognize oligosaccharides containing mannose and/or L-fucose and mediate sugar-specific endocytosis of synthetic oligolysine-based brush glycoclusters. These receptors have been suggested to be utilized for facilitation of the antigen uptake by DCs and its effective presentation [23]. Several glycocluster conjugates containing a CD8+ epitope of the Melan-A/Mart-1 melanoma antigen were designed and synthesized. The glycocluster-Melan-A conjugates were prepared by coupling different glycosynthons, e.g., oligosaccharyl-pyroglutamyl-β-alanine derivatives containing either a dimannoside (Manα-(1→6)-Man) or lactoside, or Lewis oligosaccharides Le^x and Le^a, to Melan-A 16–40 peptide Ac-G^{16}HGHSYTTAE*E*^{26}*LAGIGILTV*^{35}-ILGVL^{40}KKKK, (Ac, for acetyl) containing a variant of the HLA-A2-restricted CD8+ epitope (26–35) *ELAGIGILTV* instead of EAAGIGILTV, with a tetralysine tail on the *C* terminus. The analyses using flow cytometry and confocal microscopy have shown that fluorescent Melan-A glycoclusters with either dimannoside or mixture of Le^a and Le^x (for structures see Fig. 22.1) were taken up by DCs and concentrated in acidic vesicles, contrary to lactoside glycopeptides, which were not taken up in observable quantity. Tight binding of the dimannoside and Lewis–Melan-A conjugates to mannose receptor on DC-SIGN was shown by SPR. The lactose derivative was inactive. These conjugates with DCs call forth a CD8+ T-lymphocyte response and represent promising tools for the antitumor vaccines. Besides targeting DC-SIGN, dendrimeric structures could be used also for peptide vaccines combined with toll-like receptor agonists as adjuvants [4]. Multicomponent MUC1-based conjugates harboring besides per-glycosylated MUC1 VNTR glycopeptides a T-cell helper peptide and the TLR2 agonist $Pam_3CysSer$ have been shown to induce a robust humoral response (high levels of IgG antibodies) in animal models without an addition of external adjuvants or requirement of liposomal preparations or carrier proteins. Antisera from the immunized animals were capable to bind (glyco)peptides presented on the surface of the MCF7 breast cancer cell line [28]. Novel glycodendrimers synthesized as potential antiviral drugs can influence DC, due to their reactivity with lectin receptors, such as DC-SIGN. So it was of interest to evaluate their toxicity to DCs. When novel globular carbosilane dendrimers with mannose groups with potential therapeutic activities have been synthesized; their toxicity toward DCs had to be tested. The results showed no cytotoxicity on both immature and mature dendritic cells using up to 10 μM concentration [20]. Besides targeting DC-SIGN, dendrimeric structures could be used also for peptide vaccines combined with toll-like receptor agonists as adjuvants [4].

18.2 Direct Effects on Tumor Cells

A promising strategy is to prepare glycodendrimeric preparations that would target tumor cells and either deliver active compounds or directly inhibit tumor cells. As an example of the first possibility, mannose containing dendrimeric porphyrins, which interact with mannose-specific receptors at the surface of cancer cells, were designed for photodynamic therapy (PDT) [16]. PDT represents a promising potential treatment against retinoblastoma. The specificity of a photosensitizer and its penetration into cancer cells are crucial for achieving tumor necrosis. So the efficacy of the photosensitizers such as porphyrin derivatives against tumor cells depend on their interactions with biological membranes. The interactions between three newly synthesized dendrimeric phenylporphyrins and monolayers or liposomes with increasing cholesterol content mimicking the retinoblastoma cell membrane have been studied and the morphology of phospholipid–cholesterol–porphyrin mixed monolayers was investigated using Brewster angle microscopy [16]. It has been demonstrated that increase in cholesterol content in the model membranes had almost no effect on the effective penetration of the drugs into the lipid layers while the structured glycodendrimeric phenylporphyrins and the presence of sugar moieties especially were crucial. The data demonstrate that glycodendrimeric porphyrins, unlike non-glycoconjugated derivative, can penetrate into lipid layers even at low lipidporphyrin ratios.

Dendrimer-based preparations have also been employed for targeting drugs specifically to neoplastic cells, since current cancer chemotherapies are dose-limited because of their side effects resulting from the cytotoxicity to normal and tumor cells. G5 PAMAM dendrimer-based multifunctional conjugate that contained folic acid (FA) as the targeting agent and methotrexate as the chemotherapeutic was synthesized. The specificity and cytotoxicity of dendrimeric agent has been evaluated *in vitro* and *in vivo*. In a coculture assay using a mixture of FA receptor-positive and deficient cell lines, the preference of FA receptor-positive cells and their selective killing has been documented. This finding was supported by *in vivo* data showing that the therapy with the conjugate preferentially targeted FA receptor-positive KB cells [24].

In another study, tetra- and hexavalent mannoside inhibitors of the pro-apoptotic, antiproliferative and cell-surface clustering effects of Con A with pentaerythritol scaffolds have been synthesized by click chemistry [8]. Further analysis was focused on their influence on membrane type 1-matrix metalloproteinase (MT1-MMP) functions in marrow-derived mesenchymal stromal cells (MSC). It has been found that MSC morphological changes caused by Con A were reversed by the tetra- and hexavalent mannosides. Antiproliferative and pro-apoptotic reversal of Con A on the MT1-MMP/glucose-6-phosphate transporter signaling axis was observed and the mannosides served also as antagonists of Con A-induced caspase-3 activity and proMMP-2 activation.

A study has been dedicated to the evaluation of glycopeptide dendrimers to serve as carriers to deliver colchicine to tumor cells. In a search for tumor cell

inhibitory agents, a large screening for an effective glycopeptide dendrimer has been performed. A 65,536-member one-bead-one-compound (OBOC) combinatorial library of β-Gal containing glycopeptide dendrimers provided a lead compound against Jurkat cancer cells with LD_{50} 1.5 μM [14]. MAGs may be considered as relatively complex structures compared to other dendrimer types. However, they are relatively easy to prepare, not only as a combinatorial library, which is not possible with other dendrimer types, but also as single purified products. In this study, screening of a 65,536-member OBOC combinatorial library of glycopeptide dendrimers of structure $((\beta\text{Gal-Tpa-})_{n+1}X^8X^7X^6X^5)_2\text{Dap}X^4X^3X^2X^1(\beta\text{-Gal-Tpa}_m$ (βGal = β-galactosyl, Tpa = thiopropionic acid, X^{8-1} = variable amino acids, Dap = L-2,3-diaminopropionic acid, $n, m = 0$, or 1 if X^8 = Lys resp. X^1 = Lys) for binding of Jurkat cells to the library beads in cell culture was followed by the synthesis of selected 27 different dendrimers for further studies. MAGs selected from the combinatorial library by an on-bead cell-binding assay showed good affinity to Jurkat cells mediated by β-thiogalactoside residues at the ends of branches. The β-galactoside groups were crucial for activity. Dendrimer (βGal-Tpa-Gly-Arg-His-Ala)$_2$Dap-Thr-Arg-His-Asp-Cys-NH_2 and related analogues have been thoroughly tested as delivery vehicles. Cell targeting was demonstrated using fluorescein conjugates. The colchicine conjugate of the dendrimer was cytotoxic with LD_{50} 1.5 μM. The fluorescein derivative of another pentagalactosylated dendrimer (βGal-Tpa)$_4$(Lys-Arg-His-Leu)$_2$Dap-Thr-Tyr-His-Lys(βGal-Tpa)-Cys) selectively bound Jurkat cell, but its colchicine conjugate lacked cytotoxicity. This absence of cytotoxicity could be attributed to the fact that the colchicine dendrimer conjugates did not bind to tubulin. The inactive glycopeptide dendrimers are thus probably degraded intracellularly and the small fragments released might display weaker affinity, as compared to colchicine itself. Mannose–cytostatic complexes display a selective entry, good activity, and low toxicity, at least partially due to their specific uptake via receptor-mediated internalization by lectin receptors on tumor cells. Therefore polysaccharide mannose-tagged drug delivery systems represent an attractive strategy for the selective delivery of anticancer agents into the tumor tissues.

Doxorubicin is one of the potent anthracycline cytostatics effective against a variety of malignancies. However, this drug has the narrow therapeutic index and displays acute and chronic toxicity when used as a free drug. Hence the fundamental prerequisite in the drug delivery is spatial delivery of doxorubicin. The tumor targeting potential of surface-tailored solid lipid nanoparticles (SLNs) loaded with doxorubicin HCl and further mannosylated has been tested [13]. It has been shown that mannosylated SLNs were capable to target doxorubicin selectively and specifically to the tumor sites with minimal side effects. The preparations were evaluated for their particle size/polydispersity index and zeta-potential analysis, as well as, for *in vitro* drug release and hemolytic toxicity. The *ex vivo* cytotoxicity and cellular uptake studies were investigated using A549 cell lines. The formulations displayed a biphasic pattern characterized by initial rapid release of the drug followed by rather slow and prolonged release. Mannose-conjugated SLNs appeared to be the least hemolytic and most suitable for sustained drug delivery. They were

also most cytotoxic and preferably taken up by A549 tumor cells, as compared to uncoated SLNs and plain doxorubicin. Pharmacokinetic studies documented improved bioavailability, half life, and mean residence time of doxorubicin upon mannose conjugation. This resulted in a capacity to be delivered at a higher concentration into the tumor mass without damaging renal and hepatic tissues.

The application of dendrimeric glycopeptides in cancer detection and prevention including anticancer vaccines was reviewed in detail elsewhere [19,25,30,31]. Here, only selected recent studies are discussed.

The dendrimer capacity to function as drug carriers (discussed in Chap. 13) makes them attractive agents that can increase the efficacy of cancer chemotherapy and simultaneously minimize its adverse effects [17]. Notably, to achieve tumor cell-specific delivery, the dendrimer–drug complexes should be stable in blood circulation and the linkages should quickly degrade when internalized into target cells.

It has been shown that drugs conjugated to dendrimers through amide linkage could be selectively released in tumor tissues or cells due to their elevated levels of proteolytic enzymes, such as cysteine, aspartyl, and serine proteases including the plasminogen activators [2, 9, 18]. Further, acidic intracellular pH in many tumor cells can also facilitate drug release from the conjugate due to the pH-labile characteristics of the amide bond and introduction of pH-sensitive linkages which can be quickly hydrolyzed in acidic environment of endosomes/lysosomes represent an attractive strategy for tumor drug delivery [21].

The cytotoxicity, antiproliferative activity, and apoptosis induction activity of two modified glycosides—digoxin and proscillaridin A—conjugated to G3 PAMAM-NH_2 dendrimer were evaluated in human breast cancer cells [29]. The experiments revealed that conjugation with the G3 PAMAM-NH_2 dendrimer enhanced the cytotoxicity of modified digoxin and proscillaridin A both in MCF7 and in MDA-MB-231 breast cancer cells and conjugate-induced apoptosis was significantly greater than apoptosis evoked by free drugs. The functionalized quantum dots have a potential as bioprobes, especially in cellular imaging and drug delivery, nanosensors, and light-emitting diodes. They can be functionalized by the anchoring of biomolecules. Triantennary, β-galactoside-capped gallamide dendron synthesis was developed [5]. A click methodology was used for an assembly of the dendron with a thiolate focal group, which can secure CdSe/ZnS core/shell nanoparticle in the center by covalent anchoring. Further, biphasic extraction system for dative/covalent ligand exchange on the surface of CdSe/ZnS nanocrystals was used. The obtained nanohybrid was highly water soluble an displayed morphology of the nanocrystal core. The nanohybrid was delivered inside HeLa, kidney, and metastatic lung cancer cells with different rates and increased efficacy associated with the cell-surface-density levels of asialoprotein receptors which suggested a receptor-mediated endocytosis. This was further supported by experiments with control CL1-1 lung cancer cells with low expression of asialoprotein receptors in which the translocation of nanohybrid into cell endosomes was achieved. These triantennary multivalent nanoprobes could be a useful tool for the study of multimeric carbohydrate interactions, as well as, for endocytic and cell adhesion

and recognition processes. Due to the fact that they remain sustainable in serum-containing medium for several days, they may also serve as a photodynamic drug carrier for apoptosis studies.

Glycodendrimers have also been found useful for development of effective radiotherapy based and so-called pretargeted radioimmunotherapy against relapsed or treatment refractory B-cell lymphomas, which are currently incurable with conventional radio- or chemotherapy [10]. This method is based on tumor cell pre-targeting, using, e.g., an antitumor IgG–streptavidin complex. This step is followed by administration of a biotinylated clearing agent to quickly remove the conjugate from the blood transporting it to the liver. To be readily recognized by hepatocytes, the conjugate usually harbors galactose molecules. Finally, radiolabeled biotin is given as a therapy [22].

Recently, a setting based on a reaction of cancer-targeting fusion proteins, which comprise four anti-CD20 single-chain Fv (scFv) fragments and streptavidin (scFv4-SAv), and a biotinylated dendrimeric *N*-acetyl-galactosamine blood clearing agent (CA 1), which were subsequently radiolabeled by DOTA–biotin derivative (a monobiotin) can provide effective therapy for lymphoma xenografts in mouse models [27]. However, endogenous biotin may affect the efficacy of this pretargeting system in patients. Therefore, a pretargeting system that employs anti-CD20 scFv4-SAv mutant fusion proteins with radioiodinated bis-biotin derivatives has been developed and tested. With this system, good localization of the radiolabel to lymphoma tumor xenografts was obtained in the presence of endogenous biotin. On the other hand, since the blood clearance reagents employed in the studies were ineffective, abnormally high levels of radioactivity were seen in other tissues. Further investigation was therefore focused on utilization of a bis-biotin-trigalactose blood clearance reagent (CA 2). Moreover, another DOTA–biotin derivative (a bis-biotin) was prepared, such that radiometals (e.g., ^{111}In, ^{90}Y, ^{177}Lu) could be used in the pretargeting protocols employing scFv4-SAv mutant fusion proteins. *In vivo* experiments in mice showed that the CA 2 removed [^{125}I]scFv4-SAv-S45A mutant fusion proteins more effectively from blood, compared to the CA 1.

Synthetic glycoconjugates, namely $(GlcNAc)_8$-G2-PAMAM (GN8P) or $(GlcNAc)_4$-calix[4]arene (GN4C), have also been studied for their ability to modulate antitumor immune responses mediated by NK cells. Cancer-associated aberrant glycosylation impairs recognition capability of NK cells or modifies recognition pattern of target cells. GN4C was found to increase the susceptibility of tumor cells to cytotoxic effect of fresh NK cells or NK-92 cells, which correlated with an increased expression of NKG2D mRNA [3]. In the NK-92 cell line, GN4C induced the synthesis of IL-2, IFN-γ, and TNF-α. Cellular signaling triggered by GN4C engaged PI3-kinase/ERK (extracellular signal-regulated kinase) but not phospholipase C-gamma/JNK (c-Jun *N*-terminal kinases) pathways. In another experiment by the same group, polyamidoamine GN8P glycoconjugate was investigated for its modulation of tumor-specific B cell responses in a B16F10 melanoma murine models. It has been shown that the GN8P treatment significantly increased IgG and particularly IgG2a response against B16F10 melanoma, leading to augmented ADCC reaction [12].

Cationic antimicrobial peptides have also been identified as antitumor agents, due to their ability to target and disrupt the integrity of cancer cell or mitochondrial membrane [15]. Recently, a series of anticancer heptapeptides (H-KKW β2,2 WKK-NH_2) containing eight different central lipophilic β2,2-amino acid building blocks, which have previously demonstrated high efficiency as scaffolds in small cationic antimicrobial peptides and peptidomimetics, have been synthesized and their toxicity against human and murine tumor cell lines has been evaluated [26]. The most potent peptides displayed IC_{50} values of 9–23 μM against human Burkitts lymphoma and murine B-cell lymphoma cells and were found nonhemolytic (EC_{50} > 200 μM, using human red blood cells). The most promising peptide displayed low toxicity, when tested against human embryonic lung cells and peripheral blood mononuclear cells.

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Chapter 19
Diagnostics, Lectin Detection and Cell–Cell Interactions

The possible biological applications cover a number of biological processes and diseases. We are going to discuss several examples of them. Glycopeptide dendrimer–carbohydrate interactions are attractive for applications of dendrimers in biology [5, 6, 8, 9, 13, 37]. Cluster of highly branched glycoconjugate macromolecules, which form envelope around every cell, is called a cells glycocalyx [15, 20–22, 28, 35]. Glycocalyx is a principal player in molecular recognition and interactions of protein receptors, such as lectins, selectins, and their carbohydrate ligands. Many biological processes are strongly influenced by interactions with the glycocalyx such as immune response, fertilization, cell–cell recognition, metastasis, microbial adhesion, inflammation, and other disease states of a cell or tissue. Glycodendrimers represent excellent tools not only for studies of these biological processes occurring on cell surfaces but also for their manipulation. A glycocalyx model based on glyco-self-assembled monolayers (glyco-SAMs) is suitable for binding assays using surface plasmon resonance (SPR) [21]. A high-throughput assay based on microarrays for optimization of glycodendrimer–lectin binding affinity was also described [29]. It covers an influence of cluster effect depending on the generation of dendrimer, type of sugar, and type of lectin.

Cell structures, signaling, and cell attachment interactions are related to the interactions between cells and their surroundings. To study a number of biological processes *in vitro* and to prepare cell culture populations of particular qualities, various cultivation, plastic surface modifications with different material choices, geometric organization, and incorporation of the bioactive molecules can be designed [19]. Dendrimer-immobilized surfaces anchoring cells, which can be modified to display single or multiple ligands, can be used for studying and sculpting of cell morphologies and functions.

One of the potential targets is modulation of intercellular interactions (cellular recognition and adhesion) mediated by carbohydrate–carbohydrate interactions (CCIs) between cell surface glycans. However, more studies are required in this field [27, 34]. For CCI studies, the glycoconjugates based on G4 PAMAM dendrimers were prepared [34] and their interactions with Langmuir monolayer containing GM3

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examined. The study revealed that the CCI was adversely affected by excessive carbohydrate valency. In the presence of calcium ions, the interaction of GM3 monolayer with lactose-functionalized dendrimers was selective. The objective of these studies was to obtain useful tools for CCI analysis *in vivo* and, moreover, to develop targeted diagnostic and antimetastatic agents. Dendrimer-based agents have also been hypothesized to serve as therapeutic tool in regenerative medicine via their applications in tissue engineering and the central nervous system [27].

Another example of a potential of highly complexed branched preparations as diagnostic tools recognizing extracellular matrix (ECM) proteins has been shown [16]. Collagens and ECM proteins provide mechanical strength to tissues and are important in cell attachment, differentiation, and migration. Many pathological states (such as angiogenesis, myocardial infarction, and atherosclerosis) are characterized by ECM degradation and turnover. Visualization of collagen networks in tissues is an important tool to study these processes. The AB5 dendritic wedge represents a well-defined, highly versatile platform for the affinity enhancement of phage-display derived collagen and other ECM protein-binding peptides by mimicking key aspects of the multivalent architecture of the phage head. These collagen targeting ligands represent alternatives for antibody- and protein-mediated targeting of collagen remodeling in a number of disease processes.

Silole-core carbosilane dendrimers peripherally functionalized with lactose have been investigated as a tool for a peanut agglutinin (PNA) sensing based on fluorescence quenching detection [14]. Hatano et al. synthesized a glycocluster peripherally functionalized with a lactose derivative possessing a silole moiety as a luminophore. The photoluminescence spectrum of this glycocluster displayed extremely strong emission at 474 nm, the intensity of which decreased by increasing the amount of PNA, a lactose-binding lectin, in the solution. No quenching was seen when wheat germ agglutinin (WGA) was used. Silole-core dendrimers peripherally functionalized with carbohydrates thus could be used for a detection of target lectins.

A library of 15 bivalent α-mannopyranosides with rigid linkers was prepared in order to evaluate the effects of inter-saccharide distances onto multivalent binding interactions with bacterial and plant lectins [2].

C-Galactopyranoside mimetics have been synthesized on various dendritic scaffolds, ensuring their multivalent presentation, through click chemistry [7]. It has been shown that this type of stable *C*-galactosides could represent efficient synthetic glycomimetics of natural α-linked oligosaccharidic inhibitors of PA-IL lectin (LecA) from a pathogen *Pseudomonas aeruginosa*. Increased avidity of the glycoconjugates was observed for tri-, hexa-, and nonavalent derivatives; the most potent exhibited dissociation constants below 500 nM, corresponding to a 400-fold increase in affinity compared to control β-Gal-*O*-Me. Experiments revealed that the size and the topology of the multivalent conjugates further supported the formation of aggregative complexes as a major multivalent binding mode.

Gold nanoparticles with covalently bound mannose-functionalized G0 PAMAM layer have been tested for the protein–carbohydrate interactions through surface energy transfer process [3]. Quantitative analysis of the binding constant of a mannose-binding Con A to a sensing gold complex in aqueous samples has been

performed. The binding constant of Con A with the complexes was shown to be 100-times higher than the binding constant values obtained in the interactions with the G2 glycodendrimer alone. These results suggest that gold nanoparticles modified with glycodendrimers can be used as biocompatible and selective carbohydrate-based surface energy transfer probes for protein recognition.

A series of dendritic β-galactopyranosides and α-mannopyranosides with a terminal amino group were synthesized and used for the functionalization of single-walled carbon nanotubes (SWNTs), a pseudo-one-dimensional nanostructure capable of carrying/displaying a large number of bioactive molecules and species in aqueous solution. They targeted the defect-derived carboxylic acid moieties on the nanotube surface [12]. The higher-order sugar dendrons were more effective in the solubilization of SWNTs. It has been shown that the nanotube apparently served as a scaffold for displaying multiple copies of the sugar molecules in pairs or quartets. Further, binding assays with pathogenic *Escherichia coli* and with *Bacillus subtilis* (a nonvirulent simulant for *Bacillus anthracis* or anthrax) were performed.

Fullerenes are spherical scaffolds that can be covered with carbohydrates and used for globular presentation of carbohydrates to proteins and thus be used for protein–carbohydrate interaction studies [26]. It has been shown that mannoses in this spherical presentation can be recognized by lectins in a multivalent manner [17,24,30]. Using a Bingel–Hirsch hexaadduct and Cu(I)-catalyzed click chemistry to conjugate the sugars onto the fullerene, the spherical support was covered with mannoses and other sugars. New fullerene hexaadducts endowed with up to 24 sugar residues having globular geometry were prepared in high yields. Con A, a model for mannose-binding lectins, was recognized by mannose but not galactose residue-containing complexes. The recognition of carbohydrates involved cluster effect.

A new class of sulfurated, semirigid, radial and low-valent glycosylated asterisk ligands that could be used both as a ligand and probe has been tested for inhibition of Con A-induced hemagglutination [36]. A complete, rapid, and sugar-dependent aggregation of Con A at such low concentrations (89 nM) has rarely been reported with low-valency clusters. DLS data indicated a cross-linking mechanism capable of aggregating Con A near the nanomolar range for the α-mannose asterisk. The mechanism underlying this effect was attributed to a cross-linking effect of the asterisk, which sequesters the lectin in a macromolecular assembly and results in an amplification of the inhibition. The effectiveness of the system, compared to other ones of similar valency, is probably given by the ability of semirigid structures to optimally adjust and present the sugar epitopes to the receptor.

Inhibition of protein (lectin/toxin)–glycan interactions has potential therapeutic outputs and the design of new inhibitors is of particular interest [1]. Cyclic decapeptide RAFTs containing four Lys can be used as rigid carriers for carbohydrate derivatives with a potential of lectin-directed therapeutic agents. The bioactivity of sugar headgroups (α-TF, β-Gal, and β-Lac) covalently attached to RAFTs was investigated for a potent biohazardous plant agglutinin, a leguminous model lectin, and three adhesion/growth-regulatory human lectins. The relative inhibitory activities of these preparations reflected the affinity of free carbohydrates. Thus the potential negative effects of the chemical modification and conjugation

were excluded. Headgroup tailoring was used to increase the ligand density from tetra- to hexadecavalency and the plant toxin and tandem-repeat-type galectin-4 were especially sensitive to modification. An ability to protect cells from lectin association and adhesion by distinct glycoclusters was screened using combination of solid-phase assays with analysis of direct cell binding.

Seven topologically isomeric calix[4]arene glycoconjugates with β-Gal and α-Man, respectively, were prepared through the synthesis of a series of alkyne-derivatized calix[4]arene precursors following by the attachment of sugar moieties by microwave-assisted Cu(I)-catalyzed azide–alkyne cycloaddition. The glycoconjugates comprised one mono-functionalized derivative, two 1,2- or 1,3-divalent regioisomers, one trivalent and three tetravalent topoisomers in the cone, and partial cone or 1,3-alternate conformations [4]. Particular preparations were tested for their capacity to bind galactose-binding lectin PA-IL derived from the opportunistic bacterium *P. aeruginosa* which is a major causative agent of lung infections in cystic fibrosis patients. The trivalent conjugate showed enhanced affinity when compared to a monosaccharide model and the tetravalent conjugates were recognized as to date the highest-affinity ligands measured by ITC displaying 800-fold increase of affinity achieved by the tetravalent molecule. These experimental data could be explained by the molecular modeling, which suggested that a multivalent molecule could efficiently chelate two of the binding sites of the tetrameric lectin. Finally, SPR experiments confirmed that this glycoconjugate is the strongest inhibitor for binding of PA-IL to galactosylated surfaces and thus represents a putative anti-adhesive agent.

Importantly, G2–G3 MAGs containing *S*-α-Man have been tested as Con A inhibitors. MAGs presenting four external thiomannosyl residues and four copies of a variable amino acid were synthesized and evaluated as Con A inhibitors [10]. Their potency, evaluated through an optimized ELLA, confirmed that incorporation of external tyrosine residues close to carbohydrates reinforced the affinity for Con A. Multivalency appeared as a key feature since inhibition properties of these glycoconjugates increased with the number of carbohydrate moieties and a relatively strong cluster effect was obtained for the octavalent G3 MAG (IC_{50} 2.9 μM). Indeed, size and amino acid composition of presented glycopeptide dendrimers greatly influence binding properties and these molecular parameters are crucial for the design of lectin inhibitors. The effect of other structural modulations, such as inclusion of variable amino acids at more internal positions of the peptide scaffold or choice of branching units, should also be considered.

The cluster effect plays a crucial role in lectin–carbohydrate interactions (Fig. 19.1). To test these effects, glycodendrimer microarray has been developed for rapid screening for binding to a series of lectins and, importantly, for an investigation of cluster effects in lectin–carbohydrate interactions [29]. A microarray of glycodendrimers was constructed by means of the chip chemistry and evaluated for binding to a series of lectins, harboring multiple carbohydrate binding sites spaced at different distances. Valencies of dendrimeric structures comprising five different carbohydrates varied from 1 to 8 and corrections were made for the valencies so that all surfaces contained the same amount of the sugar ligand. WGA,

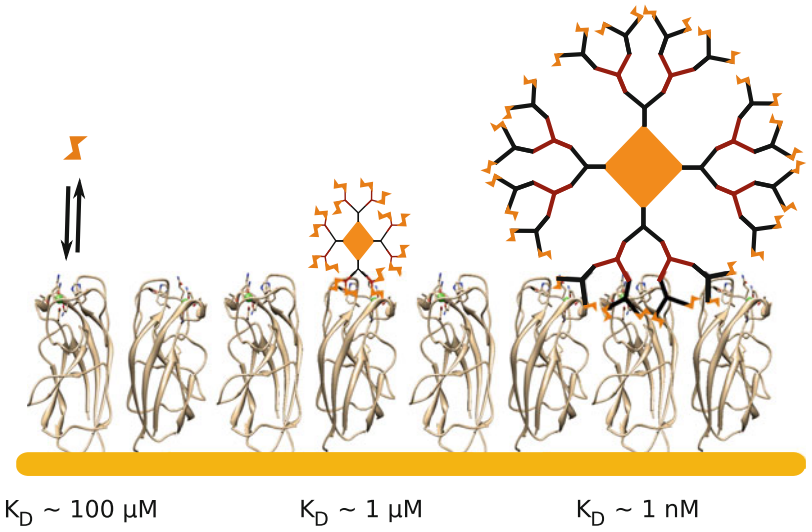

Fig. 19.1 Schematic representation of cluster effects in lectin–carbohydrate interactions. The binding affinity is amplified by clustering of many weak interactions. Adapted from [23]

a lectin containing at least two closely spaced binding sites, showed a strong cluster effect on the chip and binding was specific to GlcNAc-displaying compounds and not to the other monosaccharides, even at high valency. Cluster effects were seen also in experiments with other lectins in a few cases that correlated with the known inter-binding-site distances (smaller than about 30 Å, LecA, CTB5, WGA). On the other hand, only moderate effects were seen in cases in which inter-binding-site distances were large (i.e., larger than about 50 Å). In all experiments, the binding specificity of particular lectins was maintained even at high valency. The methods described based on real-time evaluation of a multivalent carbohydrate chip can enable to evaluate cluster effects in a single experiment.

The binding affinities of multivalent glycoconjugates toward lectins in solution are routinely measured by agglutination inhibition assays, ELISA, calorimetry, or SPR. However, these experimental designs frequently underestimate the effect derived from the lectin clustering and differences in affinity of several orders of magnitude are predicted for surface-based multivalent ligand–receptor interactions relative to those involving disperse soluble species. It has been shown that α-mannose-binding lectin Con A and three generations of clicked mannosylated gallic acid–triethylene glycol dendrimers, containing 3–27 mannose residues, can be attractive tools for mechanistic studies of multivalency due to their characteristic monodisperse nature, as well as to the possibility to control their size and branching density [25]. SPR binding experiments documented the relevance of lectin density for the reliable evaluation of binding efficiencies in surface-based multivalent carbohydrate recognition and the differences between affinity data obtained by solution and surface-based experiments. In another study, the multivalent glycodendrimer framework was evaluated as a means to describe the inhibition

potency of multivalent mannose-functionalized dendrimers using SPR [31]. Using highly robust, mannose-functionalized dithiol self-assembled monolayers on gold surfaces, glycodendrimers were found as efficient inhibitors of protein–carbohydrate interactions. The IC_{50} values for mannose-functionalized dendrimers with Con A ranged from 260 to 13 nM.

The ubiquity of lectin–carbohydrate interactions supports the notion of a glycocode (see Chap. 3), related to highly specific recognition of carbohydrate display expressed on different cell surfaces of normal and diseased origin. Synthetic carbohydrate derivatives and complexes have been used as tools for lectin–carbohydrate interactions [11]. Several symmetrical carbohydrate derivatives have been synthesized and their interaction with Con A and ultrastructural force microscopic studies of the complex formation performed. *N,N'*-Di-α-mannopyranosylurea and tris[*N,N*-α-mannopyranosyl-2-aminoethyl]amine displayed strong interactions with Con A as studied through AFM. These data suggest that small synthetic mannose conjugates could act as potential ligands for Con A binding, in contrast to previously reported highly branched mannose dendrimers.

Fluorescent carbohydrate-functionalized oligothiophenes for Con A recognition were prepared using Sonogashira-type cross coupling reactions [33]. The mannose-comprising derivatives specifically bound a model lectin Con A. It has been suggested that carbohydrate-functionalized oligothiophenes incorporated into larger dendritic structures could lead to a development of useful tools for cell-surface receptor studies and perhaps of cell adhesion inhibitors.

In the intrinsic fluorescence-based study, fluorescence lifetime experiments were performed using unlabeled lectin to characterize glycodendrimer-mediated protein aggregation. Lifetime measurements were used in these experiments to explain self-quenching phenomena induced by aggregation states [32]. Intrinsic fluorescence could be used also for more interaction studies, such as protein–protein interactions, protein–small-molecule interactions, vesicle and micelle formation, oligomerization events, and protein folding.

Glycodendrimer–lectin interactions were studied by a novel, digital, single-operation analytical method [18]. Tris(bipyridine)ruthenium(II) complexes bearing 2, 4, 6, or 18 mannose or galactose units were used as molecular logic circuits working as fluorescence devices. These devices responded to changes of pH, concentration of *N,N'*-4,4'-bis(benzyl-2-boronic acid)bipyridinium dibromide, and different lectins (Con A, GNA, and asialoglycoprotein). As output of the devices, the relative change in fluorescence and quantum yield stood for. This led to a single-step, high-throughput method for quick screening of lectin–carbohydrate interactions.

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Chapter 20
Dendrimers as Biosensors and Imaging Tools

The dendrimeric nanostructures are ideal vectors for both active and passive drug discovery, construction of biosensors, and diagnostic imaging applications. Bioactive agents might be physically adsorbed, chemically attached to the dendrimer surface, or encapsulated into the interior. This creates many options to adjust the vector properties to the specific demands of the active material and its therapeutic applications [8]. Dendrimers have been investigated as biosensors, or imaging probes for fluorescent or magnetic resonance imaging (MRI) due to their capacity to present sensing molecules on their external parts in unique, three-dimensional way. The biofunctionalization of the sensor platform deeply influences the performance of biosensors, i.e., their chemical stability, reproducibility, sensitivity, specificity, linearity, and reusability [12].

In order to develop a sensitive fluorescent sensor for lectin detection, mannose-substituted tetraphenylethenes (TPEs) were synthesized and their aggregation-induced emission (AIE) behavior induced by interactions with Con A investigated [9]. A mixture of the mannose–TPE conjugates and Con A in a buffer solution displayed an intense blue emission on agglutination within a few seconds. The sensing was selective: the conjugates act as a sensor for Con A, but not for a galactose-binding lectin, PNA. An increased sensitivity of the conjugates results if mannopyranoside substituents were linked to the TPE-core unit with a flexible chain and/or when the number of mannose residues increases.

In another study, electrochemical immunosensor signaling by employing enzyme-tagged antibody was developed in order to determine antigen or antibody under competition reaction format [6].

The enzyme-tagged antibody was prepared through covalent conjugation reaction between antibody and glucose oxidase (GOx), as an electrochemical signaling molecule. By adopting dinitrophenyl (DNP) group as a target, the conjugation reaction of GOx-tagged anti-DNP antibody was performed and confirmed by liquid chromatography. The detectable concentration range of GOx-tagged anti-DNP antibody (from 0.1 μg/ml to 0.1 mg/ml) was registered by using electrochemical signaling under competitive reaction. Target biomolecules (DNP antigen and

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anti-DNP antibody) were detected by using competitive immune reaction with the GOx-tagged anti-DNP antibody on the antigen (DNP)-functionalized sensing surface. Sensing interface based on dendrimer-assisted self-assembled monolayer was prepared and used for immunosensor measurements under competition reaction format. Target antigen and antibody exhibited linear detection ranges of 200 nM–2 mM and 1 μg/ml–0.1 mg/ml, respectively. Optimized biosensor has been developed for glucose measurements in natural samples [5]. Electroactive nanostructured membranes, prepared by the layer-by-layer technique, were utilized for preparation of electrochemical enzyme biosensors for glucose by modification with cobalt hexacyanoferrate redox mediator and immobilization of glucose oxidase enzyme. Indium tin oxide glass electrodes were modified with up to three bilayers of PAMAM dendrimers containing gold nanoparticles and poly(vinyl sulfonate). The gold nanoparticles were covered with cobalt hexacyanoferrate, a redox mediator allowing the modified electrode to be used to detect H_2O_2, the product of the oxidase enzymatic reaction, at 0.0 V versus saturated calomel electrode. The enzyme was then immobilized by cross-linking with glutaraldehyde. Several parameters for optimization of the glucose biosensor were investigated, including the number of deposited bilayers, the enzyme immobilization protocol, and the concentrations of immobilized enzyme and of the protein that was cross-linked with PAMAM. The latter was used to provide glucose oxidase with a friendly environment, in order to preserve its bioactivity. The optimized biosensor, with three bilayers, has high sensitivity and operational stability, with a detection limit of 6.1 μM and an apparent Michaelis–Menten constant of 0.20 mM and good selectivity against interferents.

A glucose biosensor based on bioactive polyglycerol (PGLD) and chitosan dendrimers (CHD) was developed [11]. PGLD or CHD were bioconjugated with glucose oxidase (GOx). Polyaniline nanotubes (PANINTs) were used as electron mediator in these complexes due to their high ability to promote electron-transfer reactions involving GOx. The PGLD–GOx and CHD–GOx were entrapped in PANINTs during template electrochemical polymerization of aniline. Both PGLD–GOx/PANINTs and CHD–GOx/PANINTs biosensors displayed a strong and stable amperometric response to glucose even at a low potential of +100 mV, as well as a good performance in glucose concentration range in human blood. Both biosensors have a linearity range between 0.02 and 10 mM, though PGLD–GOx/PANINTs was more sensitive (10.41 versus 7.04 $nA.mM^{-1}$). The authors suggested that the difference in the biosensor behavior and the high sensitivity of the PGLD–GOx/PANINTs is caused by the specific organization of GOx layer at surface of the modifier macromolecule PGLD and their distribution in PANINTs. The enzyme affinity for the substrate, K_M^{app}, remains quite good after GOx immobilization on PGLD and CHD dendrimers and entrapment of the bioconjugates in PANINTs. The use of the PGLD–GOx and CHD–GOx for construction of glucose biosensors may facilitate the fabrication of biosensors at nanoscale, promoting the integration of labile biological components into high-throughput testing instruments and at same time biocompatible biosensors for implantation. Expected superior biocompatibility has to be proven in further studies.

New "turn-on" fluorescent sensors for lectins have been designed and synthesized [10] (Fig. 20.1). Lectins added to the glycoconjugates formed specifically

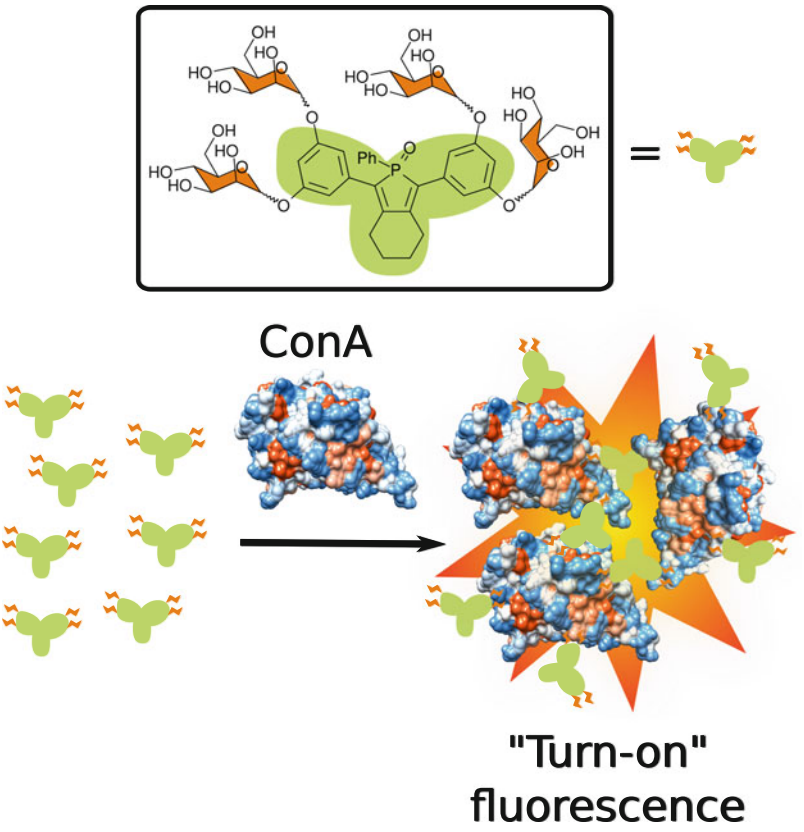

Fig. 20.1 Sugar-modified phosphole oxide served as "turn-on" fluorescent sensor of lectins with intensive blue emission. Adapted from [10]

aggregates, exhibiting an intense blue emission. This sensor assay was feasible for lectins that were multivalent or multivalently presented. Practical usefulness of the fluorescence turn-on assay has to be proven in further experiments.

The hyperbranched poly(amido amine) nanoparticles (HPAMAM NPs) with multiple functions, such as biodegradability, autofluorescence, and specific affinity, were prepared by Michael addition dispersion polymerization of *N,N'*-cystaminebisacrylamide, 1-(2-aminoethyl)piperazine, and *N*-galactosamine hydrochloride (or *N*-glucosamine hydrochloride) in a mixture of methanol/water [13]. The resultant NPs displayed strong photoluminescence, high photostability, broad absorption, and emission (from 430 to 620 nm) spectra. The fluorescence from HPAMAM NPs could be attributed to the tertiary amine chromophore. The experiments using HepG2 liver cancer cells revealed that tested fluorescent nanoparticles had low cytotoxicity and could be recognized by an asialoglycoprotein receptor on the surface of HepG2 and then internalized. This suggests that they can have potential applications in bioimaging and drug or gene delivery.

For lectin recognition based on luminescence resonance energy transfer (LRET), upconverting lanthanide Ln^{3+}-doped nanoparticles conjugated with glycodendrimers were developed [3]. PAMAM dendrimers were adsorbed on the surface of these upconverting nanoparticles (LnNPs) via direct ligand exchange. The surface modification was further tightened by thiourea linkage formation between the amine surface and p-isothiocyanatophenyl-α-mannopyranoside. These water dispersible and biocompatible mannose-coated PAMAM conjugates were used to recognize Con A conjugated with tetramethylrhodamine via LRET from the nanoparticles. Nanoparticles play a role of energy donors to the labeled lectin molecules acting as energy acceptors. The energy transfer phenomenon of the nanoparticles was induced by excitation at 980 nm, the Con A conjugate replied by emissions at 585 nm. Surface energy transfer process allows for rapid and sensitive determination of the binding constant of a mannose-binding protein [2]. In the same laboratory, ligand-free LnNPs were functionalized with heparin and basic fibroblast growth factor and used to obtain high-contrast images of HeLa cells [1].

Another dendrimeric lectin-binding sensor with up to eight mannose residues was synthesized by click chemistry [4]. The attachment of the dendrimer core to aluminum oxide chips was done via a spacer. A real-time binding of the fluorescent lectins Con A and GNA to the glycodendrimer chips was observable. In a single experiment, the multivalent enhancement or cluster effect of the binding event could be observed. Difference between small effect for Con A and large one for GNA was explained by twelve times higher density of GNA binding sites than those of Con A. Chips coated with these dendrimers served for screening of multivalent effects. Kinetic and thermodynamic data of binding and inhibition events are easily accessible.

Gd(III)-containing dendrimers represent an example of dendrimeric structures with potential to be used for MRI. Knowledge of the relative locations and concentrations of Gd(III) in dendrimers is crucial for better understanding of their toxicity and effectiveness [7]. Electron paramagnetic resonance (EPR) of a stable Gd(III) complex with diethylenetriaminepentaacetic acid (DTPA) has been studied in various PAMAM dendrimers as a function of dendrimer generation, core, and dendrimer surface functionality. The EPR spectral analysis documented anisotropic locations of Gd–DTPA inside the dendrimer and computer analysis of the EPR spectra of the probes identified the interactions of the Gd-dendrimers with ions and organic molecules. It has been shown that the interactions between the probes and the dendrimer's internal and external surface depend on the type of core, the composition of the external surface, and the generation of the dendrimer.

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Chapter 21
Dendrimers Regulating Intracellular Signaling Pathways

Dendrimers have been utilized for regulating intracellular signaling pathways. Series of dendrimers harboring tetrapeptide sequences containing an immunoreceptor tyrosine-based activation motif (ITAM) phosphotyrosine motif, which were synthesized and conjugated to dendrimers via click chemistry to create a series of functional phosphopeptide-containing dendrimers, have been shown to activate spleen tyrosine kinase (Syk) through binding of their tandem Src homology 2 (SH2) domains [3]. Syk is known to be activated through its divalent interactions of each SH2 domain to a diphosphorylated ITAM. The specific tetra- and octavalent dendrimers harboring ITAM tetrapeptide sequences have been shown to display high affinity in the nanomolar range, using surface plasmon resonance competition experiments. The effect of multivalency was demonstrated by comparative analysis in which tetra- and octavalent dendrimers displayed higher K_d than the divalent or monovalent constructs.

Bone marrow-derived mesenchymal stem cells (MSCs) represent a population of self-renewing, multipotent cells that are able to differentiate upon different stimulations into a number of phenotypes, such as osteogenic, chondrogenic, adipogenic, cardiomyogenic, and neurogenic lineages. Their commitment can be regulated by the intercellular signals. It has been found that alterations in small signaling G protein Rho family GTPase activities derived from cytoskeletal formation can lead to guidance of cardiomyogenic differentiation of human mesenchymal stem cells (hMSCs) *in vitro*. A dendrimer-immobilized substrate that displayed glucose was employed to regulate the cytoskeletal formation of hMSCs [2]. It has been shown that active migration of cells during the culture, accompanied by their morphological changes of stretching and contracting, correlated with an increase in the dendrimer generation number. The most dramatic effects on Rho family GTPase activities, RhoA down-regulation and Rac1 (another small signaling G protein of the Rac subfamily of Rho family) upregulation, were observed when the G5 dendrimer surface was used. This was associated with altered cellular morphology and migration. Cell aggregation was promoted on this surface indicating that an increase in *N*-cadherin-mediated cell–cell contacts and Wnt signaling regulates hMSC differentiation toward cardiomyocyte-like cells.

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The $P2Y_{14}$ receptor is a G protein-coupled receptor activated by uridine-5'-diphosphoglucose and other nucleotide sugars that modulate immune function. The enhanced pharmacological activity of receptor agonists upon their conjugation to PAMAM dendrimers has been demonstrated [1]. Uridine-5'-diphosphoglucuronic acid (UDPGA) and its ethylenediamine adduct were coupled to several generations (G2.5-6) of dendrimers (both carboxy and amino terminal). The compounds were coupled with prosthetic groups, such as biotin, a chelating group for metal complexation (and eventual magnetic resonance imaging), and a fluorescent moiety, for molecular detection and characterization of the $P2Y_{14}$ receptor. The activities of conjugates were investigated using HEK293 cells stably expressing the human $P2Y_{14}$ receptor. A G3 PAMAM conjugate containing 20 bound nucleotide moieties (UDPGA) was 100-fold more potent (EC_{50} 2.4 nM) than the native agonist uridine-5'-diphosphoglucose. A molecular model of this conjugate docked in the human $P2Y_{14}$ receptor showed that the nucleotide-substituted branches could extend far beyond the dimensions of the receptor and be available for multivalent docking to receptor aggregates. Larger dendrimer carriers and greater loading favored higher potency. A similar conjugate of G6 with 147 out of 256 amino groups substituted with UDPGA displayed an EC_{50} value of 0.8 nM. This study demonstrated that biological activity of the agonists was either retained or dramatically enhanced in the multivalent dendrimer conjugates in comparison with monomeric $P2Y_{14}$ receptor agonists.

Noninvasive imaging of dendrimer-type *N*-glycan clusters has been studied [4]. This study demonstrates a difference in the *in vivo* dynamics and biodistributions between $\alpha(2\rightarrow6)$ and $\alpha(2\rightarrow3)$sialosides, through the cluster effect allowing high selectivity and affinity in ligand–protein interactions. Interestingly, totally different dynamics of the *N*-glycans between the normal and tumor models were discovered. Research directed toward targeting cancer, inflammation, and immune-related organs by using the developed glycoclusters is currently under way.

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Chapter 22
Vaccines and Immunomodulation

Nanoparticles (NPs) such as dendrimers, polymeric NPs, metallic NPs, magnetic NPs, and quantum dots are under intensive study as vaccine carriers and/or adjuvants for both infectious diseases and cancer immunotherapy [3]. Antigen mannosylation is known to increase its uptake and presentation by APC. A novel mannose-based antigen delivery system utilizing a PAMAM dendrimer was used [47]. Mannosylated dendrimer ovalbumin (MDO) was shown to be a potent immune inducer since not only enhanced antigen presentation but also induction of DC maturation was observed. In a functional study, mice immunized with MDO generated strong OVA-specific CD4+/CD8+ T-cell and antibody responses associated with inhibition of B16-OVA tumor growth in vaccinated animals. These results documented adjuvant capacity of mannosylated PAMAM dendrimers when they were used as vaccine carriers.

The monocyte–macrophage (MΦ) lineage can undergo different pathways of activation. The classical priming by IFN-γ, then triggering by lipopolysaccharide, conducts MΦ toward proinflammatory responses, whereas the alternative activation by IL-4, IL-10, IL-13, or glucocorticoids directs them toward an anti-inflammatory, immunosuppressive phenotype [15]. It has been shown that synthetic phosphorus-containing dendrimers activate human monocytes. In further studies, the gene expression in monocytes activated by dendrimer with phosphazene core and phosphonic acid in the shell was compared to gene expression in untreated monocytes. It was found that 78 genes were upregulated, whereas 62 genes were downregulated. The results suggested that phosphorus-containing dendrimers induce an alternative-like, anti-inflammatory activation of human monocytes. Gene expression analysis was confirmed by quantitative real-time polymerase chain reaction and analysis of the surface expression of specific markers by flow cytometry. These results were in agreement with functional experiments of inhibition of CD4+ T-lymphocyte proliferation in mixed leukocyte reaction which indicated that dendrimer-activated monocytes (damonocytes) have an immunosuppressive phenotype similar to the one induced by IL-4 and preferentially enhanced amplification of CD4+ T cells, producing IL-10, an immunosuppressive cytokine. Phosphorus-containing dendrimers thus

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could have a potential for the treatment of uncontrolled inflammatory processes in acute or chronic diseases such as psoriasis, rheumatoid arthritis, or autoimmune diseases.

Glycodendrimers have been investigated for their capacity to increase the immunogenicity of DNA-based vaccines, e.g., by DNA delivery into professional antigen-presenting cells (APCs). The adjuvant effects of dendrimers on dendritic cells, employed for antitumor therapy, are discussed in detail in Chap. 18.1. The dendrimeric platform based on G5-PAMAM dendrimers, a DNA-loading surface, with major histocompatibility complex class II targeting peptides that can selectively deliver these dendrimers to APCs has been conjugated with DNA and transfected murine and human APCs *in vitro* [10]. Subcutaneous administration of these complexes led to preferential transfection of DC in the draining lymph nodes and promoted generation of high affinity T cells associated with rejection of established tumors.

Another approach, by which it is possible to increase antigen immunogenicity by its targeting into APC, is antigen mannosylation. Mannose-binding C-type lectins on APC surface (e.g., mannose receptor, DC-SIGN, dectin-1, or langerin) are able to facilitate mannosylated antigens uptake and presentation. Mannosylated PAMAM dendrimer, conjugated with the OVA, was highly immunogenic in mice [47], generating strong OVA specific cellular and humoral immune responses. This conjugate not only enhanced antigen presentation but also induced dendritic cell maturation. In a therapeutic experiment, immunized mice were resistant to challenge with B16-OVA tumor cells.

Besides their use as immune response activators, dendrimers can also be used as inhibitors of the immune system activation. As an example, G3.5 PAMAM dendrimer modified with glucosamine molecules was proved to inhibit lipopolysaccharide (LPS)-toll-like receptor (TLR) 4-mediated synthesis of proinflammatory and angiogenic mediators [46]. These data document that synthetic dendrimers could be used as agents preventing scar tissue formation. Recently, molecular modeling studies revealed mechanisms by which glucosamine-containing dendrimers can prevent TLR4-myeloid differentiation-2 protein-LPS complex formation [2]. This knowledge can be very important for novel TLR antagonists development.

Altogether, probably the most attractive field for utilization of dendrimers in immunology is their use as synthetic vaccines [6, 22, 41]. A number of experimental vaccines have been designed against infectious diseases and against cancer. A fully synthetic peptide vaccine based on polyacrylate dendritic polymer has been developed against *Streptococcus pyogenes* (commonly known as group A *Streptococcus*; GAS), which is responsible for acute and postinfectious complications, such as rheumatic fever and rheumatic heart disease [60]. J14 peptide, derived from the *C*-terminal region of the M protein, which has been identified as vaccine candidate, has been coupled to dendritic polymer. Intranasal administration, without additional adjuvant, induced specific IgG antibodies which could opsonize GAS *in vitro*. Although more studies are required, the data document the potential of acrylate dendrimers to be used as nasally administered vaccine against GAS. Interestingly,

PEI has been used as a DNA vaccine against intracellular pathogen *Chlamydophila psittaci*, which primarily infects birds [55]. Aerosol delivery of this construct lead to significant protection of turkeys against *Cp. psittaci* challenge.

22.1 Antitumor Vaccines

Dendrimeric structures have also been intensively studied for their capacity to mimic tumor or viral antigens and thus induce specific immunity; for review, see [6,7,41,44,53,59,61]. Glycoproteins of the outer cell membrane are endowed with a number of biological processes important for intercellular interactions and cell recognition. Aberrant glycosylation of the cell surface glycoproteins is associated not only with autoimmune diseases but also with cancer. Glycosylation, which stabilizes conformation, modifies their physicochemical properties, as well as protects them from proteolysis, plays a crucial role in protein antigenic properties and conformation [21,37,45]. Glycoconjugates with incomplete or altered glycosylation expressed on tumor cells can represent tumor-associated glycopeptide antigens or tumor-associated carbohydrate antigens (TACAs) (Fig. 22.1). They can be divided into two main classes: In *O*-glycopeptides containing carbohydrate antigens (e.g., T_N, TF, sialyl-T_N, sialyl-TF, α-2,6-sialyl-TF, α-2,3-sialyl-TF, KH-1, globo H, GM1, fucosyl-GM1, GM2, GM3, GD2, GD3, Le^y (Fig. 22.1)), the sugars are bound to OH groups in the side chains of Ser or Thr while *N*-glycopeptides containing carbohydrate antigens are characterized by the sialyl-Le^x and sialyl-Le^a bound to the amide group of Asn [16,36,41]. Importantly, TACAs cannot be considered only as tumor markers, but they are responsible for some typical features of tumor cells, such as metastasis development or invasiveness [20,23].

In the first generation of vaccines, TACAs were conjugated to carrier proteins such as KLH, BSA, or OSA. However, these conjugates induced low level of the desired antibodies, irrelevant response against the carrier due to undefined composition of the molecule and low molecular ratio of the antigens over the carrier protein. Thus a novel generation of fully synthetic vaccines, e.g. based on dendrimeric lysine scaffold, namely MAP or MAG, was developed and used as nonimmunogenic carrier for B-cell antigens and T-cell helper peptides [5]. Multivalency of synthetic antigens is crucial for recognition of the epitopic structures by specific antibodies and for subsequent cluster formation. For example, the T_N antigen is recognized by specific MLS 128 mAb, which is composed of three or four consecutive GalNAc-serines/threonines [40]. The topic of TACAs and development of synthetic antitumor carbohydrate vaccines and their activities have been reviewed; see [4,13,14,23,24,26,28,29,33,34,39,42,43,48,49,58].

Tetrameric vaccines designed for multiple antigen presentation were prepared by conjugation of a Lys_2Lys MAP core with four molecules of glycododecapeptide antigen from the tandem-repeat sequence of the tumor-associated mucin, MUC1, harboring a sialyl T_N-antigen saccharide side chain [30]. Second tetrameric vaccine prepared by the same method comprised a T cell epitope from tetanus toxoid and

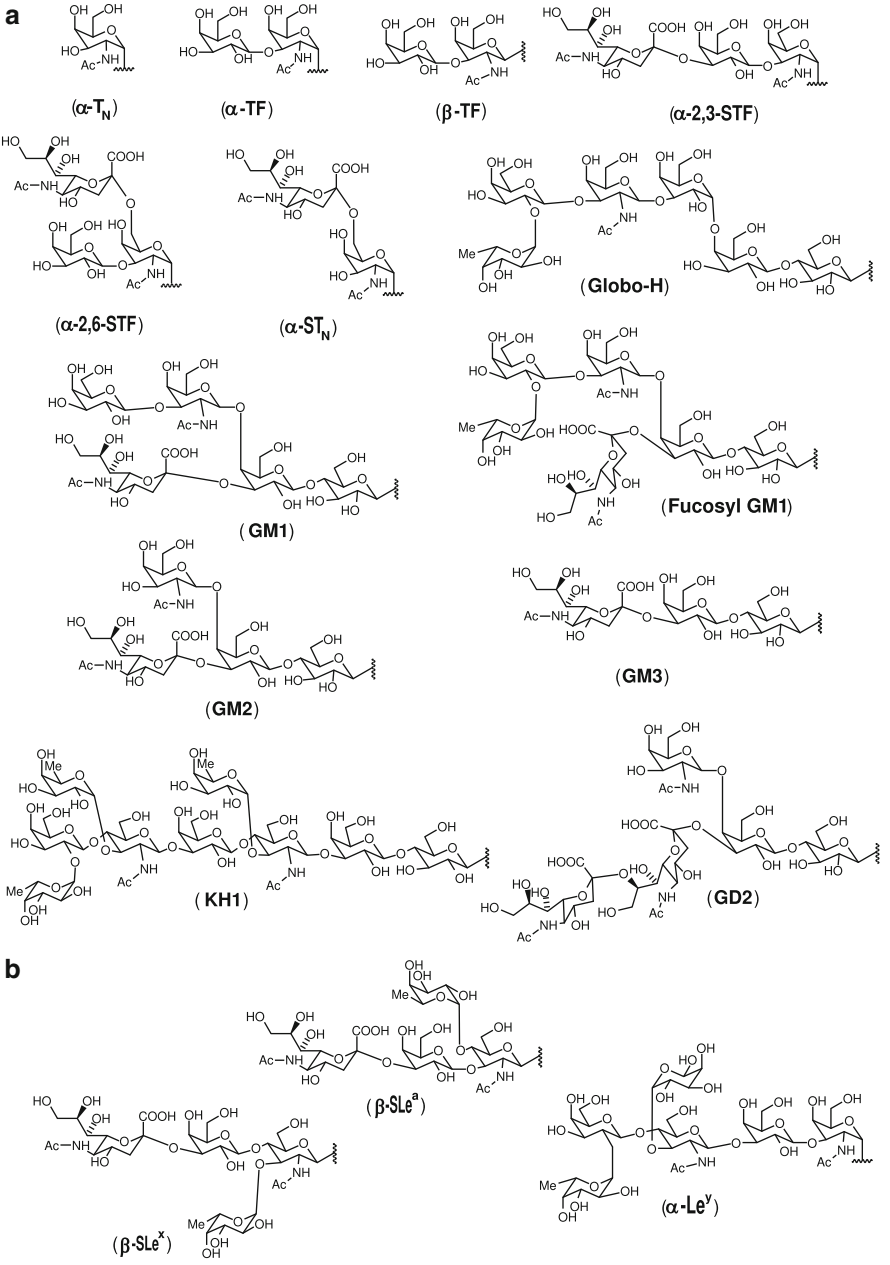

Fig. 22.1 Different types of tumor-associated carbohydrate antigens [1, 4, 11, 14, 16, 21, 25, 29, 31, 32, 36, 41, 42, 50, 56]. (**a**) *O*-glycopeptides (**b**) *N*-glycopeptides

the sialyl T_N glycododecapeptide of MUC1. The size of the resulted constructs (molecular weight of about 12,000) suggests that they could induce immune responses without being bound to carrier proteins.

Several dendrimeric preparations have been already tested in clinical trials, such as vaccines against breast [18, 19], prostate [51], and small cell lung cancers [32]. Immunologic responses have been observed in those studies. In the case of prostate cancer, antitumor effect of α-GalNAc-*O*-serine/threonine conjugate vaccine was demonstrated.

22.2 Antiviral Vaccines

Dendrimer-based-antiviral carbohydrate vaccines can be designed to target heavily glycosylated viral envelope glycoproteins, such as the HIV-1 gp41 and gp120. Antibody 2G12, isolated from a long-term survivor of HIV infection, recognizes either high-mannose or hybrid-type glycans on Asn 332, 339, and 392 residues of gp120, is able to efficiently neutralize a wide spectrum of different HIV isolates *in vitro* and to protect macaques from simian–human immunodeficiency virus challenge [54]. The constructs mimicking the 2G12 epitope could thus be a potential antigen candidate for HIV vaccines. The synthesis and antibody-binding affinity of a cholic acid template-assembled oligomannose cluster as an epitope mimic for human anti-HIV antibody 2G12 have been described [12, 35, 57]. These synthetic glycoconjugates represent potential immunogens capable to induce carbohydrate-specific neutralizing antibodies against HIV-1. These results demonstrate that selected glycosylation patterns of the HIV viral protein gp120 probably can themselves serve as epitopes for potent, broadly neutralizing Abs. Danishefsky's group [17, 38] has reported the chemical synthesis of gp120 glycopeptide high-mannose-type fragments gp120(316-335) and gp120(331-335). The triantennary undecasaccharide was assembled by two efficient methods and then conjugated with gp120 peptide segments through direct aspartylation. However, more research has to be performed in the field. In another study from the same laboratory, series of glycopeptides harboring the conserved oligomannose structure $Man_9GlcNAc_2$, which represents an epitope recognized by the broadly neutralizing human mAb 2G12, was prepared by coupling to a cyclic peptide scaffold [27]. SPR studies revealed that divalent and trivalent, but not monovalent, compounds were capable of binding 2G12. However, when these conjugates were coupled to protein carrier and two animal species were immunized, poor recognition of HIV gp160 by induced antibodies was observed.

Another strategy using dendrimeric peptide vaccines has also been employed for a development of antiviral vaccines. An anti-HIV peptide vaccine comprising B-cell epitope (15 amino acids (317–331) of the V3 region of HIV-1, JY1 isolate (subtype D) in tandem with a T-helper epitope corresponding to the 830–844 region of tetanus toxoid was synthesized in several preparations, including oligomerization, MAP format, dendrimers, and conjugation to dextran beads or to other macromolecular carriers and tested in mice. The dendrimer preparation of the peptide

conjugated to HBsAg protein was a better immunogen than the dendrimer alone and showed a higher immunogenicity than other multimeric presentations or than the peptide alone conjugated to dextran. Moreover, the dendrimer, either alone or conjugated to HBsAg, enhanced cross-reactivity toward heterologous V3 sequences as compared to monomeric peptide [8]. More recently, the use of a dendrimeric peptide to protect pigs against challenge with foot-and-mouth disease virus (FMDV) has been described [9]. Pigs, which were immunized intramuscularly with a MAP containing one copy of a FMDV T-cell epitope at the *C* terminus and branching out into four copies of a B-cell epitope at the Lys branches, specifically displayed high titers of FMDV-neutralizing antibodies and activated FMDV-specific T cells and did not develop significant clinical signs upon FMDV challenge.

Recently, dendrimeric peptide vaccine candidates for classical swine fever virus were described [52]. Each dendrimeric construct contained four copies of a B-cell epitope from the E2 glycoprotein of CSFV [construct 1: E2 (694–712); 2: E2 (712–727); 3: E2 (829–842)] joined to the amino groups of tetravalent MAP. The T-cell epitope from the NS3 protein (residues 1446–1460) was on the carboxy terminus of the dendrimer. Pig immunization with these constructs significantly reduced the clinical score after lethal challenge with CSFV.

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Chapter 23
Dendrimers in Neurodegenerative Diseases

This chapter deals with applications of dendrimers for treatment of Alzheimer's (AD) and Parkinson's diseases (PD), as well as the diseases which involve the prion proteins such as scrapie, bovine spongiform encephalopathy (BSE), Gerstmann–Straüssler–Scheinker's syndrome (GSS), Creutzfeldt–Jakob's disease (CJD), and its new variant (nvCJD). These neurodegenerative diseases include at least one stage where the formation of amyloidogenic plaques takes place [39, 43, 49]. The accumulation of peptides or proteins with propensity to β-sheet aggregation leads to viscous gel formation and suppression of cellular trophic functions. The neurodegenerative diseases are accompanied with cell damage caused by accumulation of these toxic and pathological proteins [42]. In AD and PD, the aggregation of Aβ peptide and α-synuclein is related to its organism of origin [43], whereas, in prion diseases, it is believed that the misfolded protein is a causative agent of transmissible encephalopathies and can act in an organism different from that of its origin [1, 22, 49]. Whether the seed propagation is responsible for disease transmission is still not clear [43]. Recently, the interactions between Aβ and prions have been described and attributed to certain role during the pathogenesis [7, 36].

23.1 Dendrimers as Anti-prion Agents

In prion-related neurodegenerative diseases, the cellular prion protein (PrP^C) is converted from soluble α-helical rich protein to partially insoluble, β-sheet rich, and resistant protein (scrapie form, PrP^{Sc}) toward proteases [1, 22, 49]. PrP^{Sc} aggregates are accumulated in neuronal tissue as a constant feature of all prion diseases. PrP^{Sc} is the sole causative agent of the prion diseases [1, 22, 49]. Natural and synthetic prion proteins were studied by X-ray fiber diffraction [73].

PrP^{Sc} is very stable toward many chemical and physical treatments [72]; however, its infectivity is completely suppressed when it is exposed to high concentrations of protein denaturants like guanidinium and thiocyanate ions [50]. Since PrP^{Sc} inactivation and dissociation proceed via a solubilization of PrP^{Sc},

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its solubilizers can serve as a suitable model for PrP^{Sc} inactivation [61]. Peptidic β-sheet breakers suppress PrP^{Sc} infectivity by interfering with the transition of PrP^{C} to PrP^{Sc} and by (partially) unfolding of PrP^{Sc} [64]. Another way of PrP^{Sc} inactivation is acidic SDS treatment [47].

Several pharmacological strategies for tackling the prion disease have been described [68]. One of them is using dendrimers. Dendrimers are key players in PrP^{Sc} deactivation [65, 66, 70]. For example, the G5-PAMAM dendrimer with 64 surface primary amino groups at 0.1 μM completely prevented PrP^{Sc}-caused toxicity on ScN2a cells. Moreover, the presence of dendrimers makes fibrils sensitive to protease K degradation. The mechanism of conversion of PrP^{Sc} into protease-sensitive form is not known. Increase of positive charge on the dendrimer surface at a low pH increases the efficiency of the PrP^{Sc} dissolution [9, 69].

The smaller model of prion protein—so called mini prion with affinity His_6-tag—has predilection for protease-resistant conformation [67]. Its resistance toward protease is strongly dependent on the length, charge, and placement of the affinity tag. PPI dendrimers rendered the mini prions sensitive to protease digestion.

Polycationic dendrimers are useful tools for sterilization of medical instruments, because they convert conformationally altered prion protein to non-infectious one [51, 52].

The proteins interact with both dendrimer interior and exterior [21]. Due to a correlation of activity with pH, the interactions were mainly attributed to electrostatics. The immobilization of denaturing groups on dendrimeric surfaces profoundly affects dendrimer–prion interactions. These denaturating dendrimers can be potentially used as reagents for prion decontamination, i.e., removal of prions from skin, surgical instruments, surfaces, etc. [54]. Synergistic accumulation of both cationic dendrimers and PrP^{Sc} in lysosomes catalyzes the acidic dendrimer-mediated PrP^{Sc} disaggregation [69]. Many different amyloid proteins can be disaggregated by dendrimers. Dendrimers not only break preformed fibers but also cap elongating fibers. Despite an excellent anti-prion activity, an application of dendrimers as therapeutics of protein misfolding is limited by poor bioavailability and detrimental neurological side effects.

Maltose-modified PPI dendrimers were prepared from unmodified G2–G5 PPI dendrimers and excess of maltose by reductive amination [28]. These dendrimers with enhanced molecular rigidity and the maltose units on the surface can form a lot of hydrogen bonds. The interactions with HuPrP(185–208) were investigated with the modified and unmodified dendrimers. The maltose-modified dendrimers are significantly less toxic and have potential to be applied as anti-prion agents. These nontoxic anti-amyloid agents with no cationic charges initiated the search for more efficient anti-prion agents.

In order to increase charge density and reduce the intrinsic cytotoxicity of polyamines, quaternization of amine groups of G4 PEI and PAMAM dendrimers was carried out [40]. For diminished cytotoxicity, the quaternized dendrimers paid with slightly reduced anti-prion activity. Besides, these dendrimers inhibited prion propagation catalyzed by conversion of PrP^{C} to PrP^{Sc} without dramatic loss of activity. The structural control of dendrimers extends space for improvements,

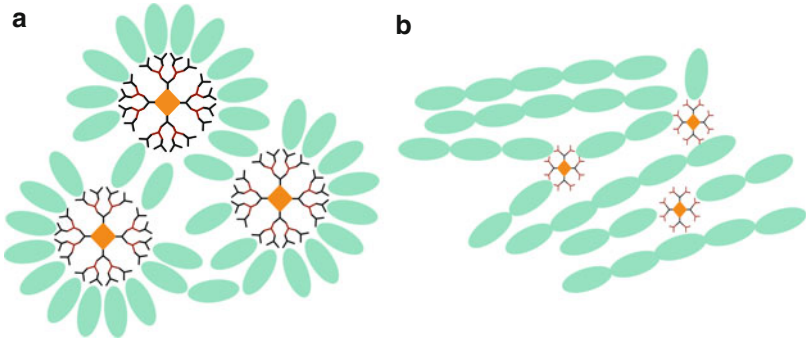

Fig. 23.1 An effect of cationic dendrimers on amyloid Aβ1-42 (**a**) the higher generation of dendrimers is sufficient in reorganizing the gel formed by Aβ1-42, whereas the small generation are only partially active in a disruption of fibril growth (**b**). Adapted from [33]

because cytotoxicity and anti-prion activity is strongly dependent on the structure of dendrimers. The quaternization is a useful strategy for preparation of non-toxic dendrimers with potent anti-prion activity.

Changes in *C*-terminal charge, oxidation state, and conformational stabilization provide large alterations of amyloidogenecity of HuPrP(106–126) [25]. A charged *C*-terminus favors amyloid formation, whereas the oxidation heavily prevents amyloidogenecity. Moreover, a perturbation of peptide fibrillation is achieved by cationic dendrimers. The perturbation depends on the kind of charge-bearing group. When two copies of HuPrP(106–126) are attached to lysine (i.e., a simplest MAP), the amyloidogenecity is completely eliminated for free acid, whereas the amide had almost the same behavior as monomeric HuPrP(106–126). It is noteworthy to say that one copy of prion peptide was acetylated. The similar suppression of amyloidogenecity was achieved by attachment of RGG motive on *C*-terminus.

Interactions of PPI dendrimers with PrP(185–208) and Aβ(1–28) depend on the level of protonation of His, Glu, and Asp residues [31], particularly when a concentration of dendrimer is low. At high concentrations, dendrimers cleared the fibrils of the both peptides. At pH 5.5, the PrP(185–208) did not aggregate. The ionization state of peptide residues is a main culprit of sensitivity to dendrimeric anti-amyloidogenic activity. For G3–G5 PAMAM dendrimers, the higher generation, the more effective clearance of amyloidogenecity of PrP(185–208) and Aβ(1–28) is [33] (Fig. 23.1).

Previously, cationic PPI dendrimers were able to abolish prion infectivity and protease resistance. Their activity was mainly attributed to the polycationic surfaces of the compounds. However, the recent study revealed that the cationic surface is not necessary for anti-prion activity [17]. PPI were modified by a reductive amination with maltose and maltotriose. The cationic layer was densely covered with sugars providing surface rich of hydroxyl groups and with buried positive charge. The glycodendrimers possessed similar activity as their cationic parents; however, their cytotoxicity was diminished. This work combines together the quaternization and introduction of glycol surface protection.

G1–G5 PPI dendrimers with guanidine- (positively charged at neutral pH) and urea-functionalized (uncharged) surfaces were synthesized [9]. The PrP^{Sc} solubilization by these dendrimers was studied in an SMB cellular system infected with the prion disease. These dendrimers and the unmodified ones possessed almost the same effect in PrP^{Sc} clearance. The anti-prion activity is almost proportional to the generation. Urea functionalization led to partially less active dendrimers (lower positive charge), which were much less cytotoxic. The higher activity of cationic guanidine- and non-functionalized PPI dendrimers is counterbalanced by their extreme cytotoxicity. The most efficient G4 and G5 PPI dendrimers were unmodified and guanidino modified, which cleared PrP^{Sc} completely at less than 50 nM concentration. Therapeutic applications of modified dendrimers can be allowed by the low effective concentrations.

Amyloid fibrils formed in both Alzheimer's and prion diseases can associate to glycosaminoglycans, modulators of the aggregation [10]. Because amyloid peptides and proteins without sequence homology form almost the same aggregates, the possible existence of a common formation mechanism was predicted. A sequence homology between PrP(185–208) and Aβ(1–28) was used for study of influence of histidine and heparin on the aggregation. The heparin induces amyloidogenesis of PrP(185–208). Histidine influences the kinetic similarly to that of Aβ(1–28); however, the magnitude is different. Both peptides are cytotoxic to a neuroblastoma cell line. The heparin induces and accelerates the aggregation of both peptides [32]. The effect of heparin is bound to the His residues in the peptide sequences. Aβ(1–28) includes VHHQKL—a heparin-binding consensus sequence—and the sequence VTHQK is a part of the *N*-terminus of PrP(185–208).

Anti-prion agents such as quinacrine, an FDA-approved drug, failed in clinical trials [8]. The failure was attributed to the formation and accumulation of quinacrine-resistant isoforms of prion protein. PAMAM dendrimers led to clearance of protease-resistant isoforms and conversion to the protease-sensitive molecules [20]. Thus a way to the combined therapy was opened. Only larger and positively charged dendrimers were able to convert resistant isoforms to protease-sensitive one. The proposed mechanism takes into account an accumulation in endosome, where both cationic dendrimers and cationic prions cannot coexist. Therefore, the formation of insoluble protease-sensitive isoform was favored. It is not known if the toxic aggregates were dismantled or just buried in benign precipitate.

Heparin can influence fibril formation in the pathogenesis of prion diseases. Hence, the interactions between dendrimers and heparin and their consequences for the anti-prion activity of dendrimers were studied [29]. Heparin can both accelerate or inhibit prion aggregation depending on its concentration. For instance, human prion peptide 185–208 (HuPrP(185–208)) aggregates in the presence of optimal amount of heparin, but reducing or increasing of concentrations by one order of magnitude did not lead to an apparent aggregation. Cationic dendrimers with anti-prion activity (PAMAM, PPI, and phosphorus dendrimers) and anionic heparin interact mainly electrostatically. These interactions can be indirectly responsible for the inhibition or enhancement of the fibrillation by dendrimers.

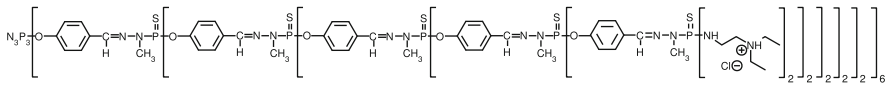

Fig. 23.2 G5 phosphorus-containing dendrimer with powerful anti-prion activity [63]

The Aβ(1–28) peptide, a model of peptide involved in Alzheimer's disease, and the HuPrP(106–126) peptide, a simplified model of prion protein, interacted with three different types of dendrimers as shown by spin-probe and spin-label techniques [30]. Aβ(1–28) interacts more strongly with dendrimers than HuPrP(106–126). PAMAM dendrimers suppress the fibrillation caused by the peptides more than PPI dendrimers do.

Cationic phosphorus-containing dendrimers with tertiary amine end groups possessed a strong anti-prion activity [63] (Fig. 23.2). Moreover, these dendrimers suppress the amount of PrP^{Sc} and reduce infectivity of prions at non-cytotoxic doses.

Cationic phosphorus dendrimers (similarly to cationic nitrogen-containing dendrimers) are inhibitors of the prion peptide PrP(185–208) aggregation. Their influence was studied by a spectrofluorometric assay with thioflavin T and Fourier-transformed infrared spectroscopy [34]. The phosphorus dendrimers interfere with PrP(185–208) aggregation by both decreasing the formation of aggregates and by reducing the amount of fibrils. Application of phosphorus cationic dendrimers against scrapie prions and Aβ(1–28) has been reviewed [3].

Self-aggregation of PrP(185–208) and Aβ(1-28) was studied by EPR with 4-octyl-dimethylammonium-2,2,6,6-tetramethylpiperidine-1-oxyl bromide used as spin probe [44]. The influence of phosphorus dendrimers on the aggregation processes was investigated. It was clearly shown that the dendrimers prevent the aggregation in the lag phase, where the nucleation occurs.

Dendrimers as therapeutic agents and their potential as anti-prion, anti-Alzheimer's, anticoagulant, antidote, anti-inflammatory, and anticancer agents were reviewed [19, 24, 55, 56, 62]. Drug delivery to brain by dendrimeric systems with emphasis on application for Alzheimer's and Parkinson's diseases has been reviewed [2]. Specific targeting to inflammatory cells in the brain has been described recently [12, 13].

23.2 Dendrimers and Alzheimer's Disease

AD is accompanied with progressive and irreversible damage of memory, thought, and language [16]. It is highly spread in geriatric populations over 65 years of age. Several targets for AD therapy exist using acetylcholinesterase inhibitors, antioxidants, β-sheet breakers, nerve growth factors, γ-secretase inhibitors, and vaccines against Aβ [16]. Since many targets exist, one of the favored ways of new drug design is based on multi-targeted strategy [60].

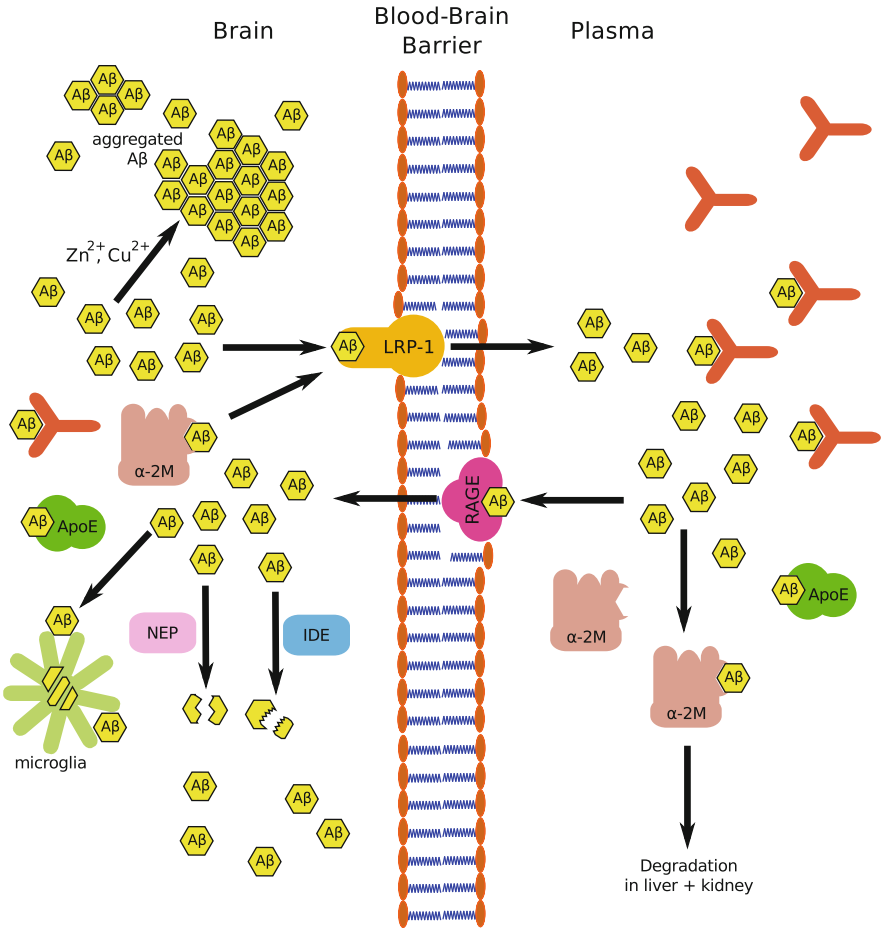

Fig. 23.3 Many roads for clearance of Aβ. Adapted from [71]

An adequate therapy for AD—the most prevalent form of neurodegeneration—is missing [38]. Aβ protein is forming senile plaques—one of the most important neuropathological hallmarks of AD. Furthermore, the Aβ oligomers are detrimental for proper neuronal function. Suppression or minimization of cerebral Aβ levels is a key aim for therapeutic strategy. Cognitive functions can be at least partially improved by application of Aβ immunotherapy by both active immunization and transfer of antibodies, respectively. There are many roads on how to clear Aβ (Fig. 23.3).

A dark spot on promising immunotherapy of AD is aborted clinical trial of AN1792 [58], probably caused by T cell-mediated immunological response. Generally, the humoral immune response is limited by aging, and immunogens and special vaccination regimes are important for an induction of a strong antibody response without an adverse immune response.

Aβ(4–10) epitope was investigated as a new vaccine against AD [41]. Bioconjugation of antigenic epitope with potential immunogens was achieved via thioether linkage of the peptide elongated by Cys or cysteinyl-pentaglycine either on *N*- or *C*-terminus. Synthetic oligopeptide carriers, such as oligotuftsin, sequential oligopeptide carrier, or lysine dendrimer-were used. The M_w of epitope conjugates was in the range of ca 4.8–9.3 kDa. In an enzyme-linked immunosorbent assay (ELISA) with an anti-Aβ(1–17) monoclonal antibody, the *N*-terminal conjugates were more effective than the *C*-terminal ones. Tetravalent conjugates with oligotuftsin, sequential oligopeptide carrier, and MAP, which were equipped with pentaglycine spacer, have nanomolar activity in the ELISA assay.

Priming with full-length Aβ(1–40)/Aβ(1–42) and subsequent boosting with MAP containing 16 copies of Aβ(1–15) provided a robust humoral immune response having only minimal T cell response [58]. The boosting with monomeric Aβ(1–15) does not provide desired immunization. Moreover, the production of anti-Aβ antibodies significantly reduces a burden caused by Aβ amyloids. These antibodies can bind monomeric, oligomeric, and fibrillar Aβ [59]. The dendrimeric carrier of the epitope can be a useful boosting immunogen for treatment of AD.

Oxidative stress is viewed as one of the primary factors triggering AD [16], especially that catalyzed by transition metals such as copper and iron via Fenton's chemistry [4, 23, 48]. In AD patients, the concentration of transition metals, as well as, the aluminum is elevated in the brain. Although the aluminum cannot undergo reductive cycling, it can facilitate oxidative damage caused by iron catalysis. Thus, the metal chelation strategy was designed as one of the active strategies against AD [16].

One of the key pathogenic events in AD is the aggregation of β-amyloid peptides into toxic aggregates [5,6,15]. Therefore, inhibition of the Aβ self-assembly process is intensively studied as one of the promising approaches. Molecules influencing this process might act as therapeutic agents for the treatment of AD. The Aβ(16–20) sequence comprising KLVFF is essential for the aggregation of Aβ and this relatively short peptide reduces amyloid deposits *in vivo* [5].

The inhibitory potential of a multimeric display of quinacrine derivatives, as compared to the monomer quinacrine, has been studied against Aβ fibril formation [15]. A multimeric conjugate exhibiting a cluster of four quinacrine derivatives on a regioselectively addressable functional template (RAFT) has been synthesized and its *in vitro* inhibitory effects against Aβ(1–40) fibrils formation were evaluated. This multimeric compound inhibited Aβ(1–40) fibril formation with an IC_{50} 20 μM, while the monomeric analogue was not active. The toxicity of this conjugate, as well as the exact mechanism of its action, needs to be elucidated. However, this compound could represent a new class of well-defined inhibitors against amyloid fibril formation in AD which should be further investigated as a possible therapeutic agent and/or as a tool to study fibril formation mechanisms.

Tetramerization of Aβ(16–20) sequence achieved by native chemical ligation of thioester with tetravalent Cys containing G1 dendrimer was used for improvement of β-sheet breaking activity [6]. The tetravalent breaker effectively inhibits the aggregation of low molecular weight and protofibrillar Aβ(1–42) into fibrils.

At the same concentration, the tetravalent system not only reduced the formation of new fibrils as the monomeric peptide does, but also it dismantled pre-formed aggregates.

Aβ-induced toxicity was attenuated by application of dendrimers coated with sialic acid [45]. Whereas the attenuation of toxicity was achieved by high doses of sialic acid, the dendrimeric sialic acid conjugates were active in micromolar concentrations (enhancements in three orders of magnitude were observed). The neuroprotective effect was attributed to sequestration of toxic Aβ by dendrimers in solution. Attachment via natural glycosidic bond provides more potent Aβ sequestrating agents than that via glycosidic linkage [46]. For amide attachment, G3 dendrimers have therapeutic index ca 140, whereas the index for G4 was approximately 50. In the case of glycosidic linkage, therapeutic indexes were 268 and 683 for G3 and G4, respectively.

Monovalent, divalent, and trivalent 6-sulfo-*N*-acetyl-D-glucosamine-immobilized substrates were prepared for the mechanistic study of amyloidosis of Aβ(1–42) [18]. Morphological images showed that Aβ(1–42) aggregates had a tendency to form globules rather than fibrils as the valency of the substrate increases. The SPR measurements showed that the morphological change of Aβ(1–42) was related to the change of binding mode, which was dependent on the multivalency of the sugar. Importantly, globular Aβ(1–42) was more toxic to HeLa cells than fibrillar Aβ(1–42). These results indicate that the multivalency of sugars for the amyloidosis of Aβ(1–42) was significant in its morphology and aggregation effects at the surface of the cell membrane mimic.

Other targets for Alzheimer's and Parkinson's disease are calpains, proteases catalyzing cleavage of particular proteins in response to Ca^{2+} signaling [11]. In order to develop their inhibitors, the calpain specificity has to be known. A degenerate MAP was used for determination of specificity at unprimed positions. The dendrimer using approach not only confirmed the known preference of calpains for hydrophobic amino acids at unprimed positions, but also a novel residue specificity for primed positions was revealed. The MER-primed site sequence directs cleavage to a specific peptide bond. A fluorescent resonance energy transfer probe with PLFAER sequence on the primed and non-primed sides was prepared. The probe was significantly more effective than the previously described ones based on α-spectrin.

23.3 Dendrimers and Parkinson's disease

Like prion and Aβ proteins are key targets of transmissible encephalopathies and AD, the α-synuclein is a culprit of PD. Hence, the inhibition of α-synuclein amyloidogenecity has a potential for PD therapy. α-Synuclein contains also hydrophobic sequence (residues 61–95) responsible for induction of apoptosis and catalysis of free radical formation [26,42]. This region is called non-amyloidogenic component.

From investigated G3.5 and G4-PAMAM dendrimers, the fourth generation can inhibit synuclein fibrillation [42], whereas the G3.5-PAMAM was ineffective in anti-fibrillation assay.

G3–G5 PAMAM dendrimers inhibited α-synuclein fibrillation [53]. The inhibition was more effective for both higher generations and higher concentrations. Structural changes of α-synuclein aggregates induced by application of PAMAM dendrimers were observed by small-angle neutron scattering. The cylindrical geometry of aggregates was converted to dense amorphous geometry. The pre-existing fibrils of α-synuclein were also broken down by PAMAMs. PAMAM dendrimers switch the pathway of aggregation from cylindrical to amorphous one.

An introduction of therapeutic approaches based on continuous dopaminergic stimulation is very important [14] for PD therapy, where the dopaminergic system is malfunctioning. The main goal of this strategy is reduction of severity of L-DOPA-associated motor fluctuations and dyskinesia, while good long-term safety and tolerability are maintained. These tasks can be tackled by application of liposomes, solid lipid nanoparticles, and biocompatible microparticles as drug delivery systems.

A key cerebroprotective agent during epilepsy and ischemia is adenosine, whose receptors are targets for treatment of diseases and disorders of central nervous system (CNS). Antagonists of A_{2A} adenosine receptor are investigated as a potential cure of PD symptoms [35]. Namely, carboxylate G3.5-PAMAM dendrimers bearing up to ten copies of adenosine mimics are potent and multivalent binders of the receptor.

Biosensors based on a quantum dot covered by dendrimeric bearers of dopamine antibodies were developed [37]. Fluorescence resonance energy transfer between the quantum dot and AlexaFluor488 was used as a signal source. It could be useful for PD diagnosis, where the dopamine level plays a crucial role in CNS.

23.4 Miscellaneous Applications of Dendrimers in Neurodegenerative Disorders

The neural cell adhesion molecule (NCAM) is important for morphogenesis of the nervous system and remodeling of neuronal connections presented in regeneration and cognition. The dendrimeric form of NCAM ligand was investigated [27]. The dendritic ligand disrupted cell adhesion mediated by NCAM induced neurite outgrowth, as well as it triggers signaling cascades analogous to those activated by homophylic NCAM binding. The ligand serves as a promoter of synaptogenesis and neuritogenesis, and as a modulator of presynaptic function of hippocampal neurons.

A fast method for tracking of protein aggregation by intrinsic fluorescence lifetime was described [57].

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Chapter 24
Conclusions and Perspectives

Dendritic macromolecules and dendrimers, pioneered in the 1980s, represent a dynamically expanding area spanning from physics, chemistry, and biology to a broad field of biomedical applications. Dendrimers interpenetrate to till now isolated areas of science and create an interconnected, logical, mutually influencing scientific network, the dendrimer science (dendrimerology). Dendrimers stimulated also the development of organic chemistry (ligations, click chemistry, and catalysis), physical chemistry (different sorts of separation methods, purification, detection, and spectroscopic methods), nanosciences (nanoparticles, nanolayers, and nano-electronics), and biomedical applications (diagnostics of microorganisms, parasites, cancer, and preparation of semisynthetic and synthetic vaccines against viruses, bacteria, and cancer). A fundamental contribution in the synthetic field has been the utilization of classical 1,3-dipolar cycloaddition, using coupling reaction of azide to alkyne with the soft and efficient copper catalyst. The use of dendrimeric libraries including dynamic combinatorial libraries lead to profitable results in both areas. One of the most attractive applications of glycodendrimers seemed to belong to bacterial and viral antiadhesins, which can protect cells from attack of pest. Glycodendrimers have enabled a better understanding and practical appreciation of the very complex nature of multivalent interactions between carbohydrates and proteins (lectins). The purity issue must be continuously considered and carefully checked, both in convergent and divergent syntheses, since consequences on functions and applications can be essential. Despite of high diversity of dendrimers, design and construction of new easily accessible monomeric building blocks will initiate development of future nanodevices.

Carbohydrate and especially glycopeptide dendrimers represent a highly sophisticated information system with unlimited pool (reservoir) of structures and corresponding functions, properties, and activities. The growing number of information about dendrimers and nanoparticles and understanding of their structures and functions led to Tomalia nanoperiodic table of self-assembled dendrons and supramolecular dendrimers, which is analogous to Mendeleev's periodic table of elements. This enables the prediction of the general features

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of tertiary structures from primary structures. The prediction reliability of the self-assembly patterns is possible in some cases with predictive accuracies of 85–90 % depending on the knowledge of the primary dendron critical nanoscale design parameters. We can say that the main aims of chemistry shifted from discoveries of elements (eighteenth century), through investigations of compounds and their reactions (nineteenth–twentieth centuries), to the research in the field of nanoscience and nanotechnology (twenty-first century). Thus, dendrimers play a crucial role of objects and tools in established nanosciences. During the last three years the number of papers about dendrimers reached about 1,600 annually, i.e., twice so much in comparison with 2001. That means that the information wave is probably at the top.

The outlook of dendrimers in general and especially peptide, glyco, glycopeptide dendrimers and analogous dendrimeric structures is very optimistic. We suppose development of new nanomaterials with tailor-made properties. Another great topic is immunology. In spite of great achievements of humankind in nuclear energy and discovering the universe, tens of millions of people are dying annually as a results of viral, bacterial, and parasitic infections and different sorts of cancer. Therefore, there is a categorical demand to develop fully synthetic, effective, vectorized, and selective vaccines, devoid of side effects. The way, including synthesis and basic chemical and immunological principles, was paved. We suppose that during the next 20–30 years the above-mentioned goal will be more or less achieved.

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