Journal of Peptide Science
J. Peptide Sci. **11**: 757–788 (2005)
Published online 29 September 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.721

JOURNAL OF PEPTIDE SCIENCE

Review

Peptide dendrimers‡

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Received 18 July 2005; Accepted 21 July 2005

Abstract: Dendrimers are branched structures and represent a fast growing field covering many areas of chemistry. Various types of dendrimers differing in composition and structure are mentioned, together with their practical use spanning from catalysis, transport vehicles to synthetic vaccines. The main stress is given to peptide dendrimers, namely, multiple antigenic peptides (MAPs). Their synthesis, physicochemical properties, biological activities, etc. have been described with many examples. MAPs can be used as diagnostics, mimetics, for complexation of different cations, as vaccines against parasites, bacteria, viruses, etc. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: multiple antigen peptides (MAPs); peptide dendrimers; synthetic vaccine; multiple antigenic peptides

INTRODUCTION

The word dendrimer stems from the Greek word *dendron* meaning 'tree' or 'branch', and *meros* meaning part [1,2].

Dendrimers are diverse branched compounds with different composition, structure, molecular weight, surface groups, valency, physicochemical properties and biological activities. The definitions depend on the specialization of the author. The more exact the definition, the more limited the area of dendrimers that belong to it. Some of the definitions are as follows:

Dendrimers are well-defined hyperbranched macromolecules with characteristic globular structures [3].

Dendrimers are versatile, derivatizable, well-defined, compartmentalized chemical polymers with sizes and physicochemical properties resembling those of biomolecules, e.g. proteins [2].

Dendrimers are highly branched three-dimensional macromolecules with highly controlled structures, a single molecular weight, a large number of controllable 'peripheral' functionalities and a tendency to adopt a globular shape once a certain size is reached [4].

Dendritic structures emerged as a new class of polymers, first reported by Vogtle *et al.*, and was named *cascade* molecules [5]. Development of this field led to larger dendritic structures and this class of compounds was renamed as *dendrimers* [2,6–8].

Our review is focused on peptide dendrimers, especially MAPs. This review is a continuation of our two earlier reviews [9,10] from 1999, a review by Sadler and Tam [11] from 2002 and the one by Tam from 2004 [12]. Therefore, we do not explain all terms, methods of syntheses, properties, etc., and the reader is referred to the aforementioned reviews. We have included literature since 2000, and in special cases, some earlier ones.

Different Types of Dendrimers

Depending on the composition and structure of the core, branches and surface groups and their substitution, we can distinguish, for example, poly(amidoamine) (PAMAM) dendrimers, also called *starburst dendrimers* (they were the first complete dendrimer family to be synthesized, characterized and commercialized [13–17], poly(propylene imine) dendrimers [13,17–19], polyester dendrimers [20], poly(propylene oxide)–polyglycerol dendrimers [21], porphyrine dendrimers with pyrimidine units [22], metallodendrimers [19,23–26], organometallic dendrimers [27–30], ferrocenyl dendrimers [23,31], Cu(II) bipyridyl-glycoclusters [32], sugar-containing carbosilane dendrimers [33], chiral dendrimers [3,18,34], silicon-based dendrimers (carbosilane, polysilane, alternating silicon–germanium, carbosilazane, siloxane and carbosiloxane [35]), siloxane and carbosiloxane dendrimers with the silicon atoms as the branching points [30,36–39], liquid crystalline dendrimers and dendrimers organized at interfaces [40,41], transition-metal-containing carbosilane dendrimers [30,42,43], phosphine-functionalized carbosilane dendrimers [44], phosphorus-containing

Abbreviations: Standard abbreviations have been followed throughout this paper (*J. Peptide Sci.* 2003; **9**: 1–8). Other abbreviations and notations used are listed in Table 1.

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‡ Dedicated to Professor R. B. Merrifield, winner of the Nobel Prize for Chemistry, on the occasion of his 85th birthday.

BIOGRAPHIES

Jan Ježek was born in 1951 in Strážnice, Czechoslovakia. He graduated from The Charles University, Prague, in 1974, and then joined The Institute of Organic Chemistry and Biochemistry of The Academy of Sciences in Prague, where he worked on both the synthesis and structure–activity studies in the newly established area of muramyl glycopeptides, under the supervision of M. Zaoral. He has worked since then at The Shemyakin Institute of Bioorganic Chemistry, Moscow, with V.T. Ivanov and T.M. Andronova (synthesis of oligosaccharide muramyl peptides and lipoglycopeptides); at The Rockefeller University, New York, with R.B. Merrifield (glucagon analogues); at The Torrey Pines Institute for Molecular Studies, San Diego, with R.A. Houghten (simultaneous multiple peptide synthesis, T-bag method) and at The Institute for Biochemistry and Biophysics, Friedrich Schiller University, Jena, with S. Reissmann (bradykinin analogues with backbone-to-backbone cyclization, synthesis and structure–activity studies). He remains at the Prague Institute, where his current interests are SPPS, MAPs, MAGs, protecting groups, coupling reagents, synthetic peptide and glycopeptide vaccines. His private interests are philately, body building and wrestling.

Petr Niederhafner was born in 1966 in Mlada Boleslav, Czechoslovakia. After graduating from the Institute of Chemical Technology, Prague, in 1988, he taught at secondary school level (1990–1996) and then went on to do research in industry (1996–2000). Since 2000 he has been at the Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences, Prague (supervisor Jan Hlavacek, PhD), working on peptide synthesis, PEG carriers and synthetic vaccines.

Jaroslav Šebestík was born in 1977 in Ostrava, Czechoslovakia. He took his degree in 2000 from the Institute of Chemical Technology, Prague. Since then, he has been a student there and at The Institute of Organic Chemistry and Biochemistry of The Academy of Sciences in Prague.

Table 1 Abbreviations and Notations Used

AAA	Amino acid analysis
Amp	*cis*-4-Amino-L-proline
APS	Antiphospholipid syndrome
Ara h 2	The major peanut allergen
CCR5 and CXCR4	The main coreceptors for cellular entry of HIV-1
CSP	Circumsporozoite protein
CTL	Cytotoxic T lymphocytes
DTH	Delayed-type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECL2	Extracellular loop 2
EGFR	Epidermal growth factor receptor
EIA	Enzyme-linked immunoassay
EIAV	Equine infectious anemia virus
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ESAT	Early secretory antigenic target
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
FL	Fluorescein
GbpB	Glucan-binding protein B
gG2	Glycoprotein G2
HA	Hemagglutinin
HAV	Hepatitis A virus
HBsAg	Hepatitis B surface antigen
HEL	Hen egg lysozyme
HHV	Human herpes virus
HLA	Human leucocyte antigen
HSV	Herpes simplex virus
IBDV	Infectious bursal disease virus
IFN	Interferon
Imd	Imidazolidine-2-carboxylic acid
IRIV	Immunopotentiating reconstituted influenza virosomes
KLH	Keyhole limpet hemocyanine
LCP	Lipid core peptide
LSA-1	Liver stage antigen-1
MHC	Major histocompatibility complex
MLV liposomes	Multilamellar liposomes
MOG	Myelin oligodendrocyte glycoprotein
NA	Neuraminidase
P_3C	*N*-Palmitoyl-*S*-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-(*R*)-cysteine
P_3CSS	Tripalmitoyl-*S*-glyceryl-cysteinyl-seryl-seryl-
PCM	Paracoccidioidomycosis
PE	1-Palmitoyl-3-oleoyl-phosphatidylethanolamine
Pn14	Pneumococcal capsular polysaccharide type 14
PrPc	Cellular prion protein
PrPSc	Scrapie prion protein
QS-21	An investigational immune adjuvant
SG3PDH	Schistosoma mansoni glyceraldehyde 3-phosphate dehydrogenase

dendrimers [19,30,45–47], cyclophosphazene-based polymers [48], phenylene-based dendrimers [49,50], peptide-functionalized polyphenylene dendrimers [51],

Table 1 (*Continued*)

SLE	Systemic lupus erythematosus
sLea	Neolactoseries antigens sialyl-Lewis a
sLex	Neolactoseries antigens sialyl-Lewis x
SP	Solid phase
TAAs	Tumor-associated antigens
TT	Tetanus toxoid
UPA	Undecapeptidyl arch

polyaromatic dendrimers with repetitive amide–ester sequence [52], peptide dendrimers (multiple antigen peptides, MAPs) [11,12,53,54] and glycopeptide dendrimers (multiple antigen glycopeptides, MAGs) [9,55,56]. Different types of dendrimers including their applications have been reviewed by Vogtle *et al.* [57].

Physical, Chemical and Biological Properties of Dendrimers

The properties of dendrimers are very diverse. Dendrimers can be used for design and construction of molecular-level systems that are capable of transferring, switching, collecting, storing, and amplifying light signals, and in information processing [18,24,50] as electroactive materials [23,58,59.], etc. Some dendrimers show photo- and electroluminescence [18,50], they can serve as pseudo-stationary phases for separations in electrokinetic chromatography (EKC) [60–63], and as nanoscopic containers and delivery devices [64,65]. Dendrimers can be used to extract dyes either in liquid–liquid systems or liquid–solid systems (dyeing of fibers) and have found a number of applications in ink-jet printing and related techniques [66]. Dendrimers with high thermal stability are also known [47]. Dendrimers can be used for catalysis [3,27,44,45,67], for asymmetric catalysis [34,43], as surfactants [21], as materials for host–guest chemistry, where molecular recognition may occur within the dendrimer interior or at its surface [13,18,39,40,68], for therapy and prophylaxis of inhaled biological toxins [69], for delivery of therapeutic oligonucleotides or drugs to tumors [70–72], for delivery of antisense oligonucleotides [73], as model compounds for DNA complexation [51], as magnetic resonance imaging contrast agents [4,74], for neutron capture therapy [2,18,75,76], in immunodiagnostics, synthetic vaccines [2,4,11,15,16,77], drug and gene delivery vehicles [2,4,11,13–16,77], etc.

Definition of Terms and Nomenclature

Dendrimers are polymers with three distinct structural features: a central core, surface functionalities and branching units that link the two [11]. The core is the central unit (in most cases symmetrical) with 2, 3, 4, etc. functional groups (amino, hydroxy, carboxy,

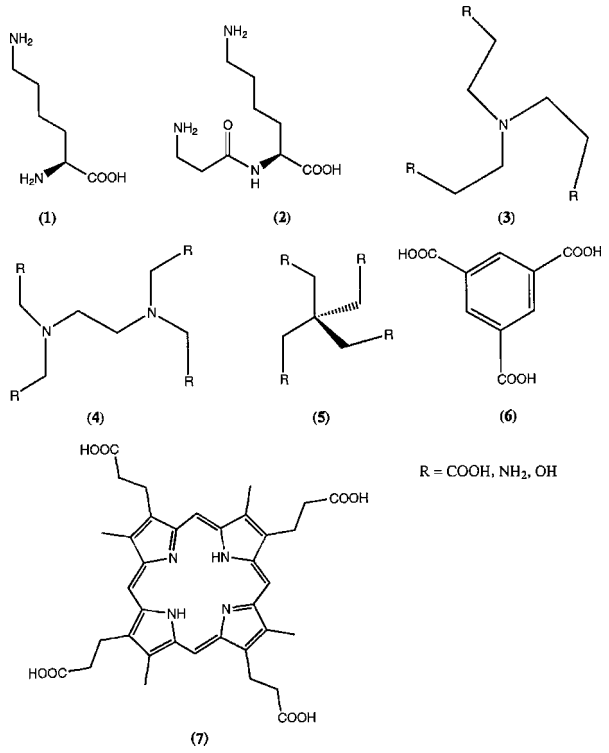


Figure 1 Examples of different amino, hydroxy and carboxy types of dendrimer cores [11,12].

etc.) to which the branches are bound. The structure of some cores are shown in Figure 1. The branches are composed of building blocks with at least three functional groups. One is bound to the core or previous generation and the second and third serve for branching to the next generation (with only two functional groups, no branching would be possible). Different structures of dendrimers and branches are shown in Figure 2. The repeating layers of building blocks in the branches are called *generations*. The exact numbering of generations has been the subject of some confusion [78]. Boas *et al.* [2] define the dendrimer generation as the number of focal points (cascade points) when going from the core to the surface; a generation 5 (G5) dendrimer thus has five cascade points between the core and the surface [79]. The core is sometimes denoted generation 'zero' (G0), as no cascade points are present (Figure 3). For a PAMAM 'Starburst™' dendrimer, for example, the core is ammonia (hydrogen substituents are not considered a focal point). Tam *et al.* [11,12] use for peptide dendrimers (MAPs) other numbering of generations. The first Lys is numbered generation 0 (G0) (Figure 3). Dendrimers, in general, and peptide dendrimers (MAPs) can be with or without a core. With the core, the structure grows into more directions, depending on the valency of the core and building units of the branches. Dendrimers and peptide dendrimers (MAPs) without the core grow only in one direction and are named *dendrons* (Figure 4) [2,11]. MAPs in most cases consist of branching units and surface functional groups.

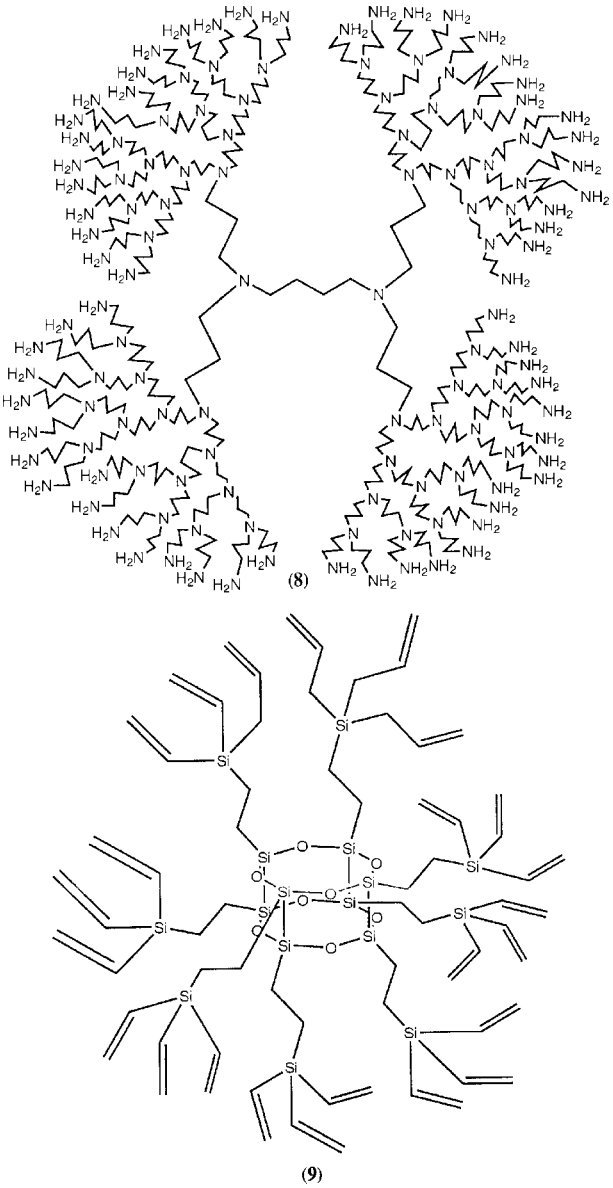


Figure 2 Different structures of dendrimers: poly(propyleneimine) dendrimer **8** and silicone-based dendrimer **9** [2].

They should be termed *dendrons* rather than classical dendrimers. MAPs can be without a core, as described by Tam [53], or with a core. The core is in most cases symmetrical, so that the branches are equivalent. MAP with symmetrical cores with core valency two or more grow in more directions and should therefore be termed *peptide dendrimers*. Use of two different terms, i.e. *peptide dendrimers* or *peptide dendrons* for analogous structures would lead to confusion. Tam [11] refers to all branched polypeptide constructs as dendrimers. We will also use this term in our review.

MULTIPLE ANTIGEN PEPTIDES (MAPs)

In this review we focus on peptide dendrimers, especially MAPs. Peptide dendrimers [11,12,54] are radial

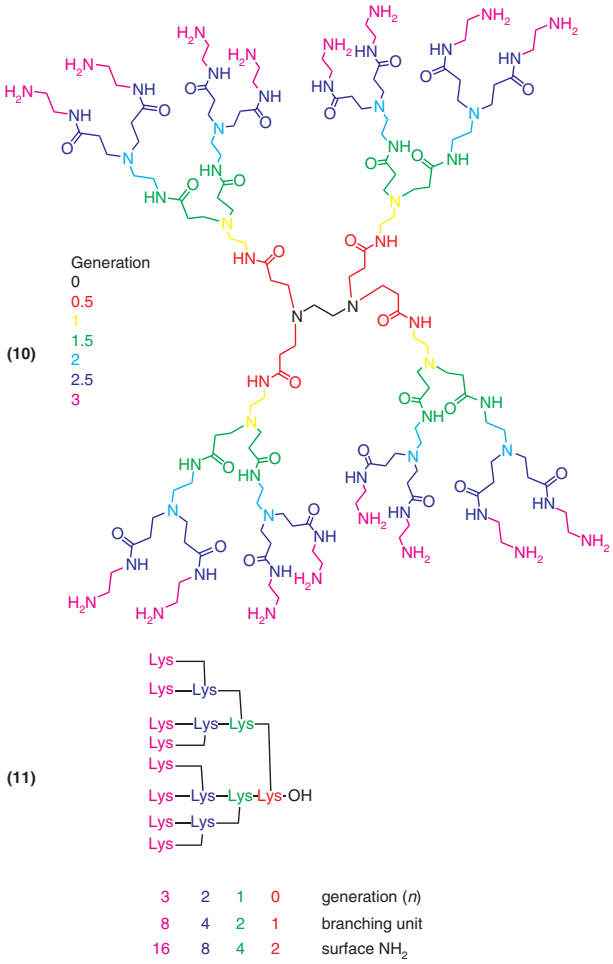


Figure 3 Definition of generation: polyamido amine dendrimer (G3) **10** [2] and MAP (G3) **11** [11,12].

or wedge-like branched macromolecules consisting of a peptidyl branching core and/or covalently attached surface functional units. Peptide dendrimers can be broadly defined as any dendrimer that contains peptide bonds. This definition would, in theory, include a dendrimer with an amino acid core, branching units, surface functional groups or any combination of the three as a peptide dendrimer. Peptide dendrimers can be divided into three types. The first are grafted peptide dendrimers. Peptide dendrimers of the second type are essentially branching polyamino acids. The third type consisting of mostly peptides has been traditionally known as *peptide dendrimers*. The most known examples of this group are MAPs [11,12,53]. Their branches consist of amino acids, and peptides are bound to the surface amino groups (or more generally the functional groups).

Unfortunately, many authors use different names for MAPs and it is difficult to understand what they mean, when an unusual term (or in combination with MAP) is used. Some examples: *multiantigen peptide* [80], *multibranched lysine core* = MAP [81], *multiantigen peptide* [82] in the title of the article, but *multiple*

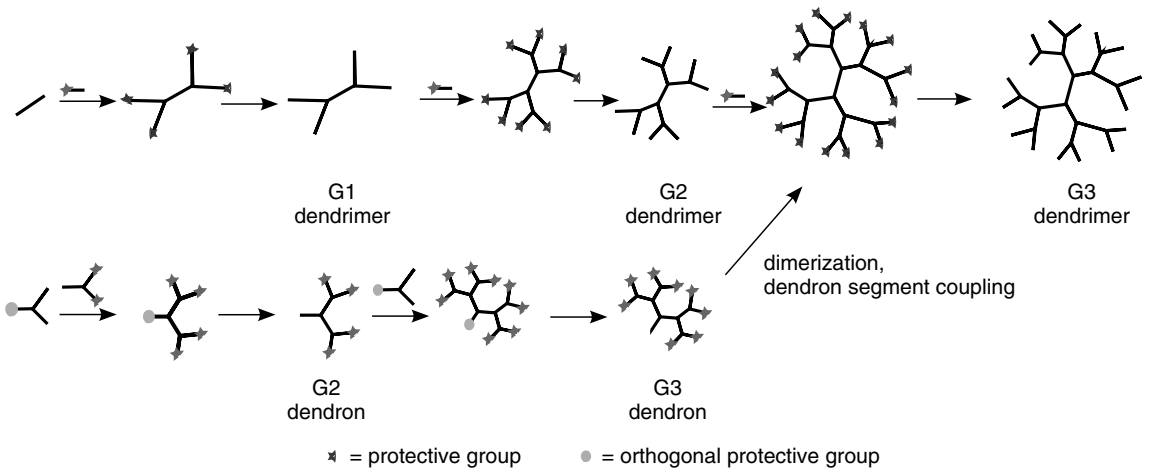


Figure 4 Dendrons and dendrimers [2,11].

antigen peptide in the text, or *multiantigenic peptide* in the title of the article but *multiple antigen peptide* in the text [83], *double dimer peptide constructs* and also *multiple antigen peptide constructs* [84] (Figure 5). Lipid core peptide (LCP) has been used for lipidic MAP [85] (Figure 5). We will use the term multiple antigen peptide (MAP).

We propose the term *tetravalent MAP* for a MAP with four identical peptides. When two different peptides are bound, it is a tetravalent bis-diepitopic MAP, etc.

Synthesis of MAPs

These compounds can be synthesized stepwise in solution, stepwise on the solid phase (divergent strategy), by fragment condensation or by ligation of prepurified fragments (convergent strategy) [11,12,54,86]. In the beginning, mainly SPPS was used for MAP synthesis. With growing complexity of the prepared compounds and problems connected with their purification and characterization, more and more ligation strategies have been used. In these cases, the fragments can be prepared in solution or SP, purified and then condensed with the branched MAP (also prepurified). There are many types of ligations, both natural (native), where a true peptide bond is created, or thiol, hydrazone, oxime etc. ligations. This topic has been reviewed [11,12,87–89] and will not be discussed here.

In most cases, MAPs have branches from lysine [11,86], but proline MAPs (Figure 6) [90,91] and ornithine MAPs (Figure 7) [92] are also known. For a review on polyglutamic, valine, leucine, arginine and depsipeptide dendrimers see Ref. 54.

Solid phase synthesis of second generation polyproline dendrimers has been reported by Albericio *et al.* [91] (Figure 6). The pyrrolidine ring of proline confers rigidity on the chain and the secondary amine decreases the reactivity during condensation reaction. The interest in synthesis of proline-rich peptides is growing, because they have an important role in many

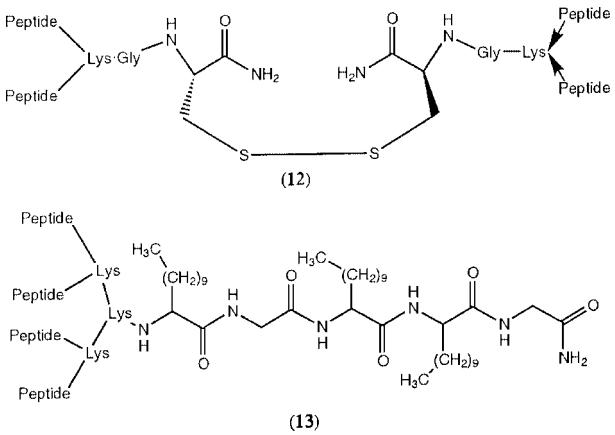


Figure 5 Double dimer construct **12** [84] and lipid core peptide **13** [85].

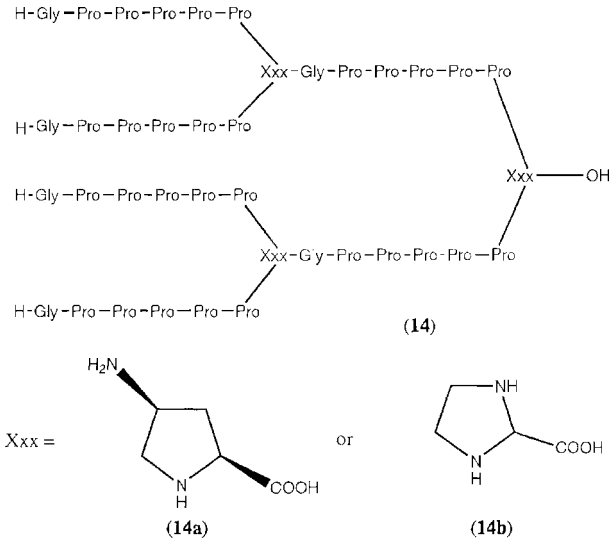
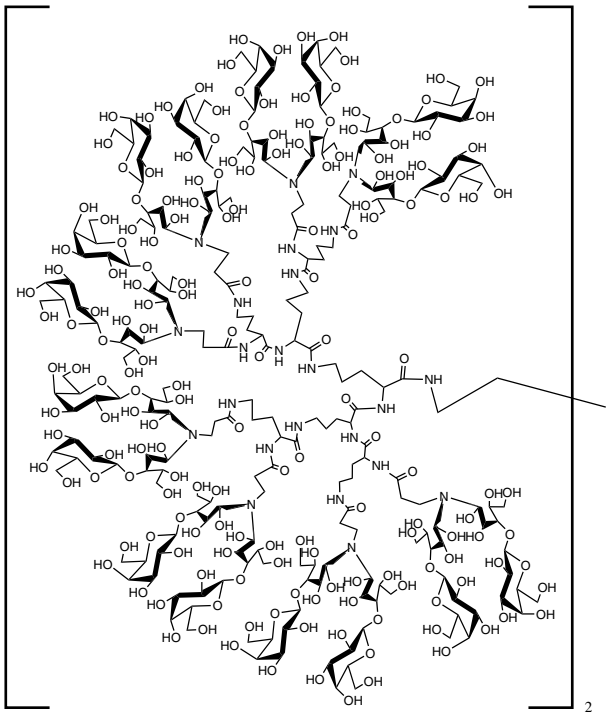


Figure 6 Proline-rich MAPs **14** [90,91].



(15)

Figure 7 Ornithine-based MAP (MAG) with 1,4-diaminobutane core containing 32 lactose units **15** [92].

biological processes such as cell motility, signal transduction, transcription and immune response. Proline-rich peptides have also features of collagens. For fragment condensations, pentaproline sequences were chosen in order to achieve proline-rich building blocks without high synthetic cost. Nonnatural amino acids *cis*-4-amino-L-proline (**14a**) (Amp) and imidazolidine-2-carboxylic acid (**14b**) (Imd) have been selected as branching units in order to minimize the possible distortions of the polyproline conformations. Besides, these amino acids have close structural relationship with proline. Imd has the additional advantage of being symmetrical and it can therefore be used either as a branching unit or as a core. The desired compounds $[(Y\text{-}Pro_5)_2\text{-}Xxx]_2\text{-}(Pro_5)_2\text{-}Xxx\text{-}OH$, where Y = Ac or H and Xxx = Amp or Imd, were obtained through a convergent SPPS approach. The method involved the synthesis of building blocks using a divergent approach (stepwise peptide synthesis starting from the core) followed by the assembly of proline-rich MAPs by convergent peptide growth. The substitution of the resin on which the fragment condensations have been done was of utmost importance. Loadings of 0.12 and 0.19 mmol/g gave 30 and 26%, respectively, of the product (Xxx = Amp; Y = Ac). When the loading was 0.68 mmol/g, the target product was not obtained at all. Because Pro, owing to its poor reactivity, is not recommended in the *N*-terminal position, to which the fragments should be coupled, Gly was incorporated at this position. Compounds $[(H\text{-}Gly\text{-}Pro_5)_2\text{-}Amp]_2\text{-}(Gly\text{-}Pro_5)_2\text{-}Amp\text{-}CONH_2$ and $[(H\text{-}Gly\text{-}Pro_5)_2\text{-}Imd]_2\text{-}(Gly\text{-}Pro_5)_2\text{-}Imd\text{-}CONH_2$ (Figure 6) have been prepared in 88 and 90% yield, respectively. Crucial for the success were low substitution of the resin and the presence of Gly at the *N*-terminus of the amino component. This provided the desired compounds with a high level of purity without the need for a further purification step. For other proline MAPs, see section on Biological Activities.

MAGs containing ornithine instead of lysine have been prepared too [92]. We include the MAGs here, because the ornithine branches are usable also for MAPs, and they contain two sugar substituents on one amino group obtained by reductive alkylation, a method usable also with peptides.

Reactions in solution have been used to prepare $(\beta\text{-}Ala_8\text{-}Lys_4\text{-}Lys_2\text{-}Lys\text{-}NH\text{-}CH_2\text{-}CH_2\text{-})_2$. β-Ala has been introduced in order to equalize the reactivity of the 16 amino groups. Reductive alkylation with borane–pyridine complex in the presence of excess of maltose, lactose, cellobiose, maltotriose or a mixture of lactose and maltose afforded MAGs with 32 oligosaccharide residues $(sugar_{16}\text{-}\beta\text{-}Ala_8\text{-}Lys_4\text{-}Lys_2\text{-}Lys\text{-}NH\text{-}CH_2\text{-}CH_2\text{-})_2$, where sugar is C1 reduced maltose, lactose, cellobiose, maltotriose or a mixture of lactose and maltose, respectively. That means, two oligosaccharides are bound to one amino group. The final purification was done by dialysis. No significant difference in reactivity was observed among maltose, lactose, cellobiose or maltotriose when a strong reducing agent borane–pyridine complex was used [93]. To distinguish the dendrimer (MAG) obtained in this study from the hemisphere-type dendrimers (MAGs) consisting of a monodendron previously reported by Uryu *et al.* [94], it is designated as a *sphere-type dendrimer* consisting of a didendron (i.e. the branches grow from the core in two opposite directions). NMR spectra support the given structure. MALDI-TOF-MS afforded experimental values 13 472.5 Da (maltose-MAG), 13 507.28 Da (lactose-MAG), 13 418.36 Da (cellobiose-MAG) and 13 384.36 Da(lactose/maltose-MAG). The calculated value for all these compounds is 13 453.87 Da. Although the mass value was 18.63 (for maltose-MAG) to 53.41 Da (lactose-MAG) higher or 35.51 Da (cellobiose-MAG) lower than the calculated value, the authors assumed that such mass differences might be attributed to errors usually occurring from salt, solvent, matrix, desorption/ionization efficiency and the detector response [93]. The final goal of the synthesis of these dendrimers was the synthesis of a glycopeptide-type human immunodeficiency virus (HIV) vaccine by binding an antigen from HIV components to the dendrimer surface. No biological data were given.

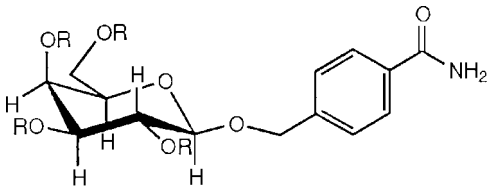
Analogous hemisphere-type oligosaccharide $Lys_4\text{-}Lys_2\text{-}Lys$ MAGs (without β-Ala) have been prepared. Since the eight amino groups included in the dendrimer had different reactivities, the number of oligosaccharide

residues connected to the dendrimer ranged from 13 to 16 [94].

MAGs containing ornithine instead of lysine with lactose or maltose as antigen have been prepared in the frame of a synthetic AIDS vaccine study [92]. The following reactions have been carried out in solution. Hemispherical ornithine dendrimer generation 2 Orn_4-Orn_2-Orn-β-Ala-OH has been prepared and used to investigate the reactivity during reductive alkylation with lactose or maltose by means of borane–pyridine complex. When the molar ratio of maltose to the amino group was in the range of 2 : 1 to 20 : 1, the number of maltose residues bound to the dendrimer having eight amino groups was 9–15.

Spherical ornithine scaffolds with or without a β-Ala periphery [(β-Ala_8-Orn_4-Orn_2-Orn-NH-CH_2-CH_2-)$_2$ or (Orn_4-Orn_2-Orn-NH-CH_2-CH_2-)$_2$] have been synthesized starting from a 1,4-diaminobutane core, followed by preparing lactose or maltose MAGs (Figure 7) [92]. The spherical MAP with 16 β-Ala on the periphery afforded MAGs with 32 molecules of reduced lactose or maltose, respectively. That means that under suitable conditions double alkylation takes place. The β-Ala was introduced in order to equalize the reactivity of the 16 amino groups. The spherical MAP without β-Ala after alkylation with maltose afforded a mixture, which, according to MALDI-TOF-MS, contained 28, 29, 30, 31 and 32 maltose residues. The properties and homogeneity of these compounds were investigated by NMR and MALDI-TOF-MS. The results of the MALDI-TOF-MS measurements were dependent on the molecular mass. For low-molecular-weight dendrimers (3–4 kDa), the found mass was in good accord with the corresponding calculated mass. As the mass increased (10.8–13.3 kDa), such as in the case of (Mal_{16}-β-Ala_8-Orn_4-Orn_2-Orn-NH-CH_2-CH_2-)$_2$ (Mal = C1 reduced maltose), a considerable difference was seen between the found (13 357.37 Da) mass and calculated (13 264.95 Da) masses. The authors argued that this might be attributed to errors occurring from the salt, solvent, matrix, desorption/ionization efficiency, selection of standard compound and detector response.

Another type of MAPs, in a broader sense, are carbopeptides [95]. They contain a carbohydrate core (template) with exactly defined orientation (in this case D-galactose) of the hydroxyl groups, to which β-Ala is bound as a spacer. Then the peptides are bound to the spacer (Figure 8). The β-hemiacetal group was protected as 4-methylenebenzoylamide (MBA-NH_2). The incorporated peptide was Ala-Leu-Ala-Lys-Leu-Gly. Carbopeptides represent the use of carbohydrates as potential templates for *de novo* design of protein models.



(16)

R = H-Ala-Leu-Ala-Lys-Leu-Gly-β-Ala-

Figure 8 MAP with D-galactose core **16** [95].

Purification and Characterization of MAPs

The methods of purification and the results obtained including characterization of MAPs depend mainly on the primary structure and the synthetic strategy used. Purification of compounds prepared by divergent strategies is more complicated, because the desired product is contaminated by thousands of deletion compounds with molecular weight, charge, polarity, hydrophilicity, etc. very similar to the desired product. The purification steps may include dialysis or gel filtration chromatography, RP-HPLC or high-performance ion-exchange chromatography. Continuous free-flow electrophoresis or other electromigration methods can be also helpful. It is important to use methods that differ in the separation principle and check their preparative efficiency by other analytical techniques. Even by these steps, it may not be possible to remove all byproducts differing by only a single modification or deletion.

MAPs and MAGs prepared by the convergent strategy are easier to purify, because the byproducts differ from the desired structure in that the whole 'arm' may be missing, and therefore the molecular weight, charge, etc. are different enough to achieve successful separation by some of the techniques mentioned above.

The first examples of successful purification and characterization of MAPs were described in 1995 [96–98] when HPLC-pure compounds fully characterized by amino acid analysis (AAA), NMR, mass spectrometry, etc were prepared. The gradient in HPLC is of utmost importance. With higher gradients (about 3%), the MAPs look pure, but at lower gradients (0.5%), the impurities are clearly seen [99].

Characterization methods include AAA (when the results agree with theory, it has low experimental value, because the product still can contain thousands of impurities), SDS-PAGE, RP-HPLC, capillary zone electrophoresis, enzymatic digestion and Edman degradation. Different kinds of NMR spectroscopy are very important, but also difficult, because the length of the arms in classical lysine MAPs is different and the measured atoms are both chemically and magnetically nonequivalent with all the consequences. The most important method is mass spectrometry (MALDI-TOF-MS, ESI-MS, etc.) [100]. MS has been used to characterize the structure of peptide mimics, MAPs and other constructs in the design of synthetic immunogens. This method determines not only the molecular weight of the product but shows also its purity. It

sometimes happens that it is not possible to obtain the correct MALDI-TOF-MS spectra (see examples of MAGs discussed previously) [92,93].

BIOLOGICAL ACTIVITIES OF MAPs

MAPs have many applications [11,12,53,54], e.g. in immunoassays, serodiagnosis, as inhibitors, mimetics, in epitope mapping and ligand binding (Table 2), cytotoxic T lymphocytes (CTL) and antibody production (Table 3). The activities are separated in the tables, but in the text they are ordered according to the associated microorganism, infectious agents or activity.

Antiparasitic Vaccines

Schistosomiasis vaccines. *Schistosomiasis* is the collective name for the clinical syndromes resulting from infection with one of the five species of schistosome

Table 2 Diagnostic and Biochemical uses of MAPs

Application	References
Drug carrier system for the antibiotic ciprofloxacin	[90]
Collagen mimetics	[101]
Transport of Na^+ across 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine liposomes as a model system	[102]
Complexation with Ca^{2+}	
Recombinant 60-kDa Ro formed precipitin lines with Ro-MAPs only in the presence of Ca^{2+} ions. The data obtained imply that Ca^{2+} induces a more native tertiary structure to recombinant 60-kDa Ro and makes it more antigenic. Thus, 60-kDa Ro is a calcium-binding protein.	[103]
Host–guest chemistry (Figure 17)	[79]
Serodiagnosis	
Hepatitis	
A method for the detection of antibodies against the hepatitis A virus (HAV) in human serum samples has been elaborated. Four synthetic constructs (one linear peptide and three MAPs) have been prepared: linear peptide VP3 (110–121) from capside protein HAV, $(VP3)_4$-MAP, $(VP3)_2(VP1)_2$-MAP and $(VP3)_2$-MAP (Figure 16). The linear VP3 and divalent $(VP3)_2$-MAP have been the most sensitive and appropriate for serological studies of sera from HAV-infected patients using BIACORE.	[104]
Hepatitis	
Fluorescence studies on the interaction of a multiple antigenic peptide from hepatitis A virus with lipid vesicles have been carried out with $(VP3)_4$-MAP.	[105]
Hepatitis	
Linear and multiple antigenic peptides have been used in the immunodiagnosis of acute hepatitis A virus infection.	[106]
Insect allatotropin immunolocalization	
Immunolocalization and possible effect of a moth allatotropin-like substance in a fly, *Phormia regina*, has been studied using MAPs.	[107]
Allergy	
MAP has been used to raise high titer antibodies and to characterize the specificity of IgE from allergenic patients sensitized to Ara h 2. The antiserum selectively detects Ara h 2 in crude peanut extract with a titer of 10^{-7} by western blot and reacts specifically with epitope 3.	[108]
Infectious bursal disease virus (IBDV)	
MAPs related to antigenic determinants of IBDV have been used for detection of anti-IBDV-specific antibody in ELISA, and quantitative comparison of MAPs with native antigen for their use in serodiagnosis has been done.	[109]
Canine EPO	
Simple and sensitive ELISA for canine EPO has been developed. This could help in developing of a commercially available assay.	[110]
HIV-1	
Comparison of tandem repeats and MAPs as the antigens to detect antibodies by enzyme immunoassay in HIV-1 model. A tetravalent MAP has been a better choice compared to the tandem repeats.	[111]
Simian immunodeficiency virus (SIV)	
EIA was developed with MAPs for the detection and discrimination of antibodies to SIV genetic lineages. The SIV MAP EIA proved to be highly sensitive and specific for detecting SIV infections in NHPs and humans. This assay has the ability to detect previously unidentified SIV strains.	[112]

Table 2 (*Continued*)

Application	References
Herpes simplex virus (HSV) MAP-based enzyme-linked immunoassays for detection of anti-HSV-2 IgG in human sera have been developed.	[113]
Comparison of different carriers The influence of carrier structure and topology on antibody recognition of attached epitope has been studied by comparing the antibody-binding properties of conjugates with tetratuftsin analogue H-(Thr-Lys-Pro-Lys-Gly)$_4$-NH_2 (T20), sequential oligopeptide carrier $(SOC)_n$, branched chain polypeptide poly[Lys(Ser_i-DL-Ala_m)] (SAK), MAP and KLH. Peptide LKNleADPNRFRGKDL ([Nle^{11}]-9-22) representing an immunodominant B-cell epitope of *herpes simplex virus* type 1 glycoprotein D has been conjugated to the above-mentioned structures via thioether or amide bond.	[114]
Human herpes virus (HHV) The mapping of the immunodominant region of the HHV-8, the antibody-binding site of glycoprotein K8.1A, has been done. The main epitope was found within residues 44–56. Tetrabranched MAP has been used as antigen to develop an enzyme immunoassay to detect HHV-8 antibodies in human sera.	[115]
Alzheimer's disease Complement C5a receptor-mediated signaling, which may be involved in neurodegeneration in Alzheimer's disease has been studied using octavalent MAPs.	[116]
Experimental autoimmune encephalomyelitis (*EAE*) EAE has been studied using myelin oligodendrocyte glycoprotein (MOG). The structure of $pMOG_{35-55}$-MAP is $(pMOG_{35-55})_8$-K_4-K_2-K-β-Ala-OH. The $pMOG_{35-55}$-MAP has been highly immunogenic and induced severe clinical symptoms even in the absence of *Bordetella pertussis* toxin.	[117]
Systemic lupus erythematosus It has been found that the murine R4A anti/dsDNA antibody cross-reacts with R4A peptide. While monomeric peptide was unable to inhibit affinity-purified polyclonal anti-DNA Abs, serum anti-DNA reactivity has been inhibited by $(DWEYSVWSLN)_8$-MAP in 10 SLE patients.	[118]
Systemic lupus erythematosus CD40 MAP has been used as the antigen in an ELISA to detect anti-CD40 MAP reactivity in the sera of APS patients and controls. The CD40 MAP was found to preferentially react with sera of patients with APS and SLE, but not with rheumatoid arthritis or normal sera.	[119]
Systemic lupus erythematosus MAPs carrying four copies of a common P0, P1 or P2 epitope have been used to verify the association of ribosomal anti-P antibodies with serological findings and clinical manifestations including neuropsychiatric involvement in a large group of 149 patients with SLE.	[120]
Systemic lupus erythematosus Tetravalent MAPs containing four copies of the *C*-terminal, 13 AAs long P-peptide have been used for diagnostic tests for antiribosomal P- protein antibodies.	[121]
Epstein–Barr virus (EBV) Three MAPs of *Epstein–Barr virus* (EBV) latent membrane protein 1 (LMP1) were used for immunization of BALB/c mice. The polyclonal antibody responses showed that MAPs evoked B-cell responses. Characterization of hybridoma supernatants showed that 11 out of 24 were IgM.	[122]
Substances affecting platelet function Lebetins from *Macrovipera lebetina* snake venom inhibit platelet aggregation induced by various agonists (e.g. thrombin, collagen). The lebetins displayed strong *in vitro* antiplatelet activity and have been able to prevent collagen-induced thrombocytopenia in rats. Several peptides have been prepared in MAP form. These MAPs have been found to be 1000-fold more active than the corresponding peptides.	[123]

adapted to man. The endemic areas, where more than 600 million people are at risk and about 200 million are actually infected, cover 74 countries in Africa, the Middle East, South America and Southeast Asia [124]. The development of the antihelmintic drug praziquantel [169] has made chemotherapy the cornerstone of control. The morbidity was dramatically reduced, but high re-infection rates even after mass treatment are limiting factors of strategies based on chemotherapy alone. Therefore, the development of synthetic vaccines is very important. MAPs with different antigens (mainly derived from the primary sequence of *Schistosoma mansoni* glyceraldehyde 3-phosphate dehydrogenase (SG3PDH)) were selected on the basis of lowest homology to human

Table 3 Utilization of MAPs for Antibody and CTL Production

Application	Reference
Parasite	
Schistosoma mansoni MAPs with different antigens (mainly derived from the primary sequence of *S. mansoni* glyceraldehyde 3-phosphate dehydrogenase SG3PDH) have been selected on the basis of lowest homology to human SG3PDH. The MAPs have been studied as possible vaccine candidates.	[99,124–127]
Schistosoma mansoni In another approach, MAPs containing peptides derived from 28-kDa glutathione *S*-transferase of *S. mansoni* have been synthesized and the antigenicities and protective effects were examined. Best results were obtained with two monoepitopic tetravalent MAPs. They seem to be potential vaccine candidates.	[128]
Plasmodium falciparum A synthetic MAP vaccine derived from *P. falciparum* circumsporozoite protein (CSP) has been tested in 39 human volunteers. The clinical side effects of the MAP vaccine trial, including local inflammatory responses and the induction of unusual local and systemic immediate hypersensitivity reactions have been tested.	[80]
Plasmodium falciparum A diepitopic MAP (T3-T1) containing two T-cell epitopes of liver stage antigen-1 (LSA-1), a diepitopic MAP containing T-cell epitopes from LSA-1 and from merozoite surface protein-1 and a triepitopic MAP (T3-CS-T1) containing T3-T1 and a potent B-cell epitope from the circumsporozoite protein central repeat region have been synthesized and tested.	[129]
Plasmodium falciparum Differential antibody responses to *P. falciparum*-derived B-cell epitopes induced by diepitopic MAPs containing different T-cell epitopes have been studied.	[130]
Plasmodium falciparum A totally synthetic polyoxime malaria vaccine containing *P. falciparum* B-cell and universal T-cell epitopes elicits immune responses in volunteers of diverse HLA types. The polyoxime MAP construct $(T1BT^*)_4$-P_3C containing repeat T- and B-cell epitopes of the *P. falciparum* (NF54 strain) CS protein has been prepared (Figure 9). *N*-palmitoyl-*S*-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-(*R*)-cysteine (P_3C) has been incorporated as an endogenous adjuvant. The $(T1BT^*)_4$-P_3C MAP vaccine was immunogenic without any exogenous adjuvant.	[131]
Plasmodium falciparum The safety and immunogenicity of an adjuvanted, synthetic *P. falciparum* CS MAP vaccine has been tested. DTH reactions have been studied as a correlate of immune response. The presence of T-cell functional activity reflected by a positive DTH skin test response to the MAP antigen served as another marker for vaccine immunogenicity.	[82]
Plasmodium falciparum A special type of tetravalent MAP (Figure 5) has been used as a possible malaria vaccine. The prepared MAPs have been immunogenic in the experimental Aotus monkey model and have been able to induce protective immunity when challenged experimentally with a highly infective *P. falciparum* strain.	[84]
Plasmodium falciparum Immune responses to MAPs containing T and B epitopes from *P. falciparum* circumsporozoite protein of Brazilian individuals naturally exposed to malaria have been tested. Three types of MAPs $(T1B)_4$-MAP, B_4-MAP, $T1_4$-MAP, and two linear peptides T1B and B have been prepared and tested. The results indicate that the ideal vaccine against *P. falciparum* malaria should be made up of multiple antigens, containing B- and T-cell epitopes.	[132]
Plasmodium falciparum Synthetic malaria MAP vaccine elicits high levels of antibodies in vaccines of defined HLA genotypes.	[133]
Plasmodium falciparum Different types of ligations for synthesis of MAPs have been studied. Tetravalent MAP prepared via thiazolidine ligation has been inoculated in rabbits to evaluate their antibody response. Titers for the tetravalent MAP were not just greater but were also sustained over time.	[134]
Plasmodium falciparum Conformationally constrained peptidomimetics in MAP form and an efficient human-compatible delivery system in synthetic vaccine design have been exploited.	[135]
Plasmodium falciparum Mimicking a conformational B-cell epitope of the heat-shock protein PfHsp70-1 antigen of *P. falciparum* using a MAP (Figure 10) has been carried out.	[136]

Table 3 (*Continued*)

Application	Reference
Plasmodium yoelii	
A subdominant $CD8^+$ CTL epitope from the *P. yoelii* circumsporozoite protein induces CTLs that eliminate infected hepatocytes from culture.	[137]
Contraception vaccines	
Cynomolgus macaques have been immunized using four adjuvants together with synthesized peptides or recombinant proteins representing selected regions of macaque PH-20 protein. The synthesized peptide (AAs 387–412, designated peptide 4) has been used as a MAP construct. The circulating antibodies from immunized animals recognized macaque sperm surface PH-20 on western blots and have been shown by indirect immunofluorescence to bind to the surface of macaque sperm.	[138]
Antitumor vaccines	
Analysis of CD8 T-cell response by IFN-γ ELISPOT and H-2L(d)/pRL1a tetramer assays in pRL1a multiple antigen peptide-immunized and RL male 1-bearing BALB/c and (BALB/cxC57BL/6) F-1 mice has been studied.	[139]
Cellular antitumor immune response directed against F98 gliomas expressing the EGFRvIII target antigen has been studied.	[140]
A unique tumor antigen peptide pRL1a, IPGLPLSL that is recognized by CTL on BALB/c RL male 1 leukemia has been identified by peptide elution. Cytotoxicity has been generated in BALB/c spleen cells by *in vivo* and *in vitro* sensitization with pRL1a peptide in the form of MAP, but not in the original form. Immunization with pRL1a MAP had a significant growth-inhibitory effect.	[141]
The antigen peptide pRL1a, present on a murine leukemia RL male 1 has been incorporated into the MAP, $(IPGLPLSL)_8$-Lys_4-Lys_2-Lys-β-Ala-OH. Immunization with the MAP generated efficiently a specific CTL response in BALB/c mice and protected the mice from RL male 1 tumor growth. The study has shown the processing of MAP in the MHC class 1 pathway and elucidated the basis for the effect of MAP as a tumor vaccine.	[81]
Inhibition of cell proliferation and induction of apoptosis by novel tetravalent MAPs inhibiting DNA binding of E2F has been studied.	[142]
$(RYDIYWRYDI)_8$-MAP has been prepared and used to immunize mice. Immunization with the MAP mimetic of sugar constituents of neolactoseries antigens induced a MHC-dependent peptide-specific cellular response that triggers IFN-γ production, correlating with IgG2a induction.	[143]
Antiviral vaccines	
HIV	
Peptides from the V3 loop of HIV-1 have been incorporated to the MAP as B-cell epitopes because of their variability. The MAPs have been conjugated to HBsAg. Mice immunized with MAP-HBsAg conjugate recognize a higher number of heterologous peptides.	[144]
HIV	
Immune response of mice immunized with MAPs containing multiple copies of either consensus or mixotope versions of the V3 loop peptide from HIV-1 bound to HBsAg have been studied.	[145]
HIV	
B-cell epitope comprising 15 AAs (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 have been used in different presentations, including oligomerization, MAPs and conjugation to dextran beads. The MAP dendrimer of the peptide conjugated to HBsAg protein has been a better immunogen than the MAP alone.	[146]
HIV	
Immunogenicity comparison of MAPs bearing V3 sequences of the HIV-1 with TAB9 protein in mice has been carried out (Figure 11).	[83]
HIV	
A cyclic dodecapeptide (cDDX4) has been conjugated with the amino groups of MAP. Immunization of BALB/c mice with cDDX4-MAP induced conformational epitope-specific antibodies. Furthermore, the antibody inhibited the replication of the HIV-1 X4 virus.	[147]
HIV	
Cyclic closed-chain dodecapeptide (cDDR5) has been conjugated to the MAP by β-COOH of Asp. The resulting cDDR5-MAP, which mimics the conformation-critical domain for HIV-1 entry, has been used as an immunogen. The cDDR5-MAP induced anti-CCR5 antibodies and inhibited infection by HIV-1 R5 in a dose-dependent manner, but did not suppress infection by HIV-1 X4.	[148]

(*continued overleaf*)

Table 3 (*Continued*)

Application	Reference
HIV The cyclic chimeric dodecapeptidyl multiple antigen peptide (cCD-MAP) has been constructed with a spacer-armed dipeptide Gly-Asp and two pentapeptides from CCR5 and CXCR4 receptors. Conformation-specific antibodies have been raised against cCD-MAP. The antibody reacted with the cells separately expressing CCR5 or CXCR4. The antibody markedly suppressed infection by X4, R5 or R5X4 virus in a dose-dependent manner.	[149]
HIV MAP derived from the conserved GPGRAF sequence present at the tip of the V3 loop of HIV envelope exerts a potent anti-HIV activity. The authors identified the target mechanism of the MAP.	[150]
HIV A MAP (PTKAKRRVVQREKR)$_4$-K$_2$-K-β-Ala-OH that encompasses the cleavage region of HIV-1 envelope precursor has been prepared. It displays an antiviral activity against HIV-1 and HIV-2 and inhibits HIV-1 Env-mediated cell-to-cell fusion.	[151]
HIV A MAP containing epitopes from the *C*-terminus of the gp120 (see above [151]) and its analogues has been used in cell-to-cell fusion assay, receptor binding assays and molecular modeling to explain the anti-HIV activity of the MAP.	[152]
HIV Carbohydrate core with branches containing maleimide has been used as a new type of template for multivalent peptide assembling. The MAPs containing peptide inhibitor T20 of HIV-1 gp41 were obtained by ligation chemistry (Figure 12). The immunogenicity of the given MAPs is currently being evaluated.	[153]
Simian immunodeficiency virus (SIV) The stimulation of SIV$_{mac}$-specific antibodies and CTL in rhesus macaques using MAPs and P$_3$CSS-peptides as immunogens has been studied. Both humoral and CTL responses were elicited. None of the monkeys were protected from infection but most demonstrated an anamnestic CTL response.	[154]
Equine infectious anemia virus (EIAV) The immunogenicity of EIAV Gag and Env equine leukocyte alloantigen (ELA)-A5.1, -A9 and -A1 restricted CTL epitopes synthesized in MAP format has been studied. The MAP has been coupled to tripalmitoyl-*S*-glycerylcysteine (P$_3$C). The P$_3$C-MAP (Figure 13) stimulated peripheral blood mononuclear cells (PBMCs) from horses, chronically infected with EIAV. The stimulated CTL lysed EIAV-infected target cells. The P$_3$C-MAP has been far better than free peptides or virus in stimulating CTL from some horses.	[155]
Measles virus The development and use of potent adjuvants for human mucosally delivered vaccines has been studied using octavalent MAP-M2 containing peptide mimic of a conformational B-cell epitope from measles F protein with sequence NIIRTKKQ.	[156]
Measles virus MAP-M2 has been compared with a chimeric peptide consisting of two copies of a T-helper epitope and one copy of the mimotope M2. The octavalent MAP-M2 induced the highest titers of anti-M2 and anti-MV antibodies. Immunization with MAP-M2 construct induced high titers of a high-affinity anti-M2 antibody despite the absence of a T-helper epitope.	[157]
Influenza Matrix protein 2 (M2) contains ectodomain (M2e) that is highly conserved among human influenza virus strains. The MAP construct containing M2e and linked helper T-cell determinants has been prepared (Figure 14). Mice inoculated twice by the *intranasal* route exhibited significant resistance against subsequent challenge with infectious virus.	[158]
Hepatitis Tetravalent bis-diepitopic [VP1 + VP3]MAP with a palmitoyl substituent in liposomes (Figure 15) elicited specific IgG not only against the MAP itself but also against the VP3 and VP1 antigens alone. The MAP constructs are suitable to be used as immunogens mimicking the native protein, and the liposome formulation of the lipophilic MAP offers a valuable strategy for the design of therapeutic vaccines.	[159]
Hepatitis Octavalent MAP (MAP 1) containing the mimotope TANGFYRLPSGS and control MAP (MAP 2) based on the epitope of AAs 130–150 of HBsAg was prepared by SPPS. The mimotope-based antigen (MAP 1) evoked higher titer of antibodies with the same specificity of the epitope-based antigen. Therefore, mimotopes can be used in antigen and vaccine design.	[160]

Table 3 (*Continued*)

Application	Reference
Antiprion antibodies	
MAPs with human prion sequences elicited in mice antibody production to the corresponding prion sequence. These peptides have been able to generate antibody responses recognizing conserved human and mouse sequences.	[161]
A fragment of PrP helix 1 (AAs 144–153 of human PrP) DYEDRYYREN was bound to octavalent MAP. A high titer of IgG antibodies against MAP-helix 1 has been obtained in BALB/C mice. It is important that purified IgG bind the whole prion protein with high affinity.	[162]
Antifungal vaccines	
Peptides derived from the 43-kDa glycoprotein (gp43) of *Paracoccidioides brasilliensis* have been used in MAP form. The MAP-induced specific lymph node cell proliferation in mice preimmunized with peptides in FCA. Besides, immunization without FCA significantly protected intratracheally infected mice. The MAP is a candidate for an anti-PCM vaccine.	[163]
Antibacterial antibodies	
Mycobacterium tuberculosis	
Efficient protection against *M. tuberculosis* by vaccination with a single subdominant epitope from the ESAT-6 antigen has been achieved using a MAP.	[164]
Pneumococcus	
A peptide mimetic (peptide 105) of the pneumococcal capsular polysaccharide type 14 (Pn14) has been studied as a model antigen to explore differences in antigenicity and immunogenicity of peptide mimotopes. The corresponding MAP competes in ELISA with native Pn14 in a concentration-dependent manner for binding to an anti-Pn14 monoclonal antibody (MAb).	[165]
Streptococcus mutans	
Peptides derived from glucan-binding protein B (GbpB) have been used to identify immunogenic regions within the GbpB sequence, suitable for use in subunit vaccines. Two *N*-terminal peptides (SYI and QGQ) subtending two of these regions have been prepared in the form of tetravalent MAPs. The MAPs have been used to subcutaneously immunize Sprague–Dawley rats. The SYI-MAP induced a higher percentage of responses to the inciting peptide as well as to the intact GbpB, as measured by ELISA. The SYI-MAP-immunized animals had significant reductions in dental caries on both smooth and occlusal surfaces.	[166]
Streptococcus mutans	
To develop broader-spectrum vaccines for dental caries, the immune potential of MAPs combining epitopes from mutans streptococcal glucosyltransferases (GTF) (epitope CAT) and glucan-binding protein B (epitope SYI) was used. Dental caries have been lower in each peptide-immunized group than in the sham-injected group, but $(SYI)_2(CAT)_2$-MAP enhanced both the immunological response to CAT and GTF epitopes and extended the protective effect to *S. mutans* and *S. sobrinus*. The addition of the GTF-derived CAT epitope to the GbpB-derived SYI peptide broadens the resulting protective immune response.	[167]
Group A streptococci (*GAS*)	
The LCP containing four copies of peptide J8 from the conserved region of the GAS M-protein has been prepared by SPPS. The LCP was lipophilized by three consecutive 2-amino-dodecanoic acids with the glycine spacers (Figure 5). The LCP construct or the J8 peptide has been used to immunize mice by the parenteral route with and without FCA.	[85]
Immunodominant T-cell epitope	
The effectiveness of synthetic peptide immunogens derived from immunodominant T-cell epitopes as replacements for their intact parent protein has been studied on hen egg lysozyme with fluorescein-labeled MAPs containing four copies of immunodominant T-cell epitope.	[168]

SG3PDH. The MAPs were studied as possible vaccine candidates [99,124–127].

In another approach, MAPs containing peptides derived from 28 kDa glutathione S-transferase of *S. mansoni* have been synthesized (octavalent monoepitopic, octavalent diepitopic, tetravalent monoepitopic and diepitopic etc.) [128] and the antigenicities and protective effects were examined. Best results were obtained with two monoepitopic tetravalent MAPs. They seem to be potential vaccine candidates.

Plasmodium vaccines. *Plasmodium falciparum* and *Plasmodium vivax* cause the majority of the approximately 300 million cases of malaria each year. The importance of *P. vivax* tends to be minimized by the extensive mortality because of *P. falciparum* in sub-Saharan Africa. However, *P. vivax* causes an estimated

80 million cases of malaria annually in South and Central America, India, Southeast Asia and Oceania [170]. The epidemiological spectrum of the disease is complicated because of drug resistance. Separate malaria vaccines must be produced to target both *P. vivax* and *P. falciparum*, as the two species are quite distinct phylogenetically and antigenically. Malaria vaccines must also be able to target the exoerythrocytic and blood-stage form of the parasite, generate both cellular and humoral immune responses, etc. Therefore, there is an urgent need to develop an effective antimalaria vaccine. Malaria is resurgent in most tropical areas because of the rapid spread of drug-resistant *P. falciparum*, the development of insecticide resistance by the Anopheles mosquito vector, population growth and the movement of nonimmune populations into malarious areas [80]. A synthetic MAP vaccine derived from *P. falciparum* circumsporozoite protein (CSP) was tested in 39 human volunteers immunized 2–3 times over 2–8 months using a dose escalation design. The clinical side effects of the MAP vaccine trial, including local inflammatory responses and the induction of unusual local and systemic immediate hypersensitivity reactions were tested.

The same group [82] tested the safety and immunogenicity of an adjuvanted, synthetic *P. falciparum* MAP vaccine. They investigated the potential for using cutaneous delayed-type hypersensitivity (DTH) reactions as a correlate of immune response. Volunteers were evaluated for DTH reactions to intradermal inoculation of several concentrations of the MAP vaccine and adjuvant control solutions. The presence of T-cell functional activity reflected by a positive DTH skin test response to the MAP antigen served as another marker for vaccine immunogenicity.

The aim of another study [130] was to compare the impact of different T-epitopes on the immune response to otherwise nonimmunogenic B epitopes. Tetravalent MAPs were synthesized as four parallel branches of a single epitope (B- or T-MAP), or two different epitopes in tandem (BT-MAP). B-epitope sequences were the *P. falciparum* circumsporozoite protein–derived $(DPNANPNV)_2$ or the Pf332 antigen-derived $(VTEEI)_3$. Pf332 is a large-sized blood-stage parasite antigen expressed on the surface of parasitized erythrocytes and it constitutes a target of opsonizing antibodies. Universal T-epitope sequences have been derived from the circumsporozoite protein CSP379-398 (IEKKIAKMEKASSVFNVVNS), CSP326-345 (EYLNKIQNSLSTEWSPCSVT), the *Clostridium tetani* toxin (QYIKANSKFIGITEL) and from the *P. falciparum* blood-stage antigen Pf332 (LVSEEIVTEEGSVAQE). C57BL/6 and BALB/c mice have been immunized with $(B–T\ diepitope)_4$-MAP containing a B-cell epitope, which is nonimmunogenic in the respective mouse strain, in combination with any of the four defined T-cell epitopes. The antibody- and T-cell responses have been analyzed. The results show that the immunogenicity of a T-epitope alone does not necessarily predict the ability of the T-epitope to provide T-cell help when combined with other epitopes in an immunogen. The nature of the immune responses in terms of total IgG antibodies and their subclass distribution, T-cell proliferation and IFN-γ production varied with the T-epitope and mouse strain. These seem to indicate the need for inclusion of combination of different universal T-epitopes in a future malaria subunit vaccine.

A special type of MAP, prepared by dimerization (DMSO oxidation) of Cys SH groups in $(peptide)_2$-KGC-NH_2 (peptide = 5 different *P. falciparum* analogous protein antigens) (Figure 5, compound **12**) has been used as a possible malaria vaccine [84]. A method was developed, named *double dimer constructs*, involving direct synthesis of a dimeric peptide with a *C*-terminal Cys-amide. This peptide has been purified by RP-HPLC. The tetrameric molecule (MAP) was obtained by oxidation of the dimeric peptide with DMSO, purified by RP-HPLC and characterized by MALDI-TOF-MS. The prepared MAPs have been immunogenic in the experimental Aotus monkey model and were able to induce protective immunity when challenged experimentally with a highly infective *P. falciparum* strain.

The same authors [134] studied different types of ligations. They used thiazolidine ligation. The tetravalent MAP containing a 45-residue monomer of the SPf-66 antimalarial vaccine was inoculated in rabbits to evaluate their antibody response. Titers for the tetravalent MAP were not just greater but were also sustained over time.

In another study, Kenney *et al.* [129] synthesized and characterized MAP conjugates containing protective epitopes from *P. falciparum* and tested their immunogenicity in four different strains of mice. A diepitope MAP (T3-T1) containing two T-cell epitopes of liver stage antigen-1 (LSA-1), a diepitope MAP containing T-cell epitopes from LSA-1 and from merozoite surface protein-1 and a triepitope MAP (T3-CS-T1) containing T3-T1 and a potent B-cell epitope from the CSP central repeat region have been tested. Peptide-specific antibodies have been produced in all four mice strains. A strong genetic restriction between the different strains of mice has been observed. In an immunofluorescence assay, anti-MAP antibodies recognized stage-specific proteins on the malaria parasites. The triepitope MAP successfully inhibited the malaria sporozoite invasion of hepatoma cells *in vitro*.

A study providing first demonstration of the immunogenicity of a precisely defined synthetic polyoxime malaria vaccine in volunteers of diverse human leukocyte antigen (HLA) types has been described [131]. The polyoxime construct $(T1BT^*)_4$-P_3C contained repeat T-

and B-cell epitopes, T1B, in combination with the universal T-cell epitope, T*, of the *P. falciparum* (NF54 strain) CSP (Figure 9). The T* epitope, representing the *C*-terminal AAs 326–345 (EYLNKIQNSLSTEWSPCSVT) has been originally identified using CD4* T cells derived from sporozoite-immunized volunteers. The polyoxime $(T1BT^*)_4$-P_3C MAP contains a 48-mer malaria sequence $(DPNANPNV)_2$-$(NANP)_3$-EYLNKIQNSLSTEWSPCSVT in each of the four branches. *N*-palmitoyl-*S*-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-(*R*)-cysteine (P_3C) has been incorporated as an endogenous adjuvant. The $(T1BT^*)_4$-P_3C MAP vaccine was immunogenic without any exogenous adjuvant thanks to the presence of P_3C. The majority of the immunized volunteers (7/10) developed high levels of antirepeat Abs that reacted with the native circumsporozoite of *P. falciparum* sporozoites. In addition, all the seven volunteers developed T-cell specific immunity for the universal epitope T*. The current phase I trial suggests that the polyoxime vaccine may prove useful for the development of multicomponent, highly immunogenic synthetic vaccines against malaria.

The dominant antibody target of the malaria parasite *P. falciparum* is the 45-kDa CSP. It includes a central region containing 41 tandem repeats of a tetrapeptide, 37 of which are NANP and 4 of which are NVDP. A cyclic mimetic of NANP motif has been coupled through a succinate linker to phosphatidylethanolamine (PE, 1-palmitoyl-3-oleoyl-phosphatidylethanolamine). The cyclic mimetic of NANP was also incorporated into the MAP, $(NANP\ mimetic)_4$-K_2-K-AAQYIKANSKFIGITELA-OH. Both the PE construct and the MAP were tested bound to alum or IRIVs (immunopotentiating reconstituted influenza virosomes). The hemagglutinin (HA) membrane glycoprotein of the influenza virus plays a key role in the mode of action of IRIVs. This antigen is a membrane-fusion-inducing component, which facilitates antigen delivery to immunocompetent cells. The authors [135] have shown that the IRIV delivery system with PE construct induced superior antisporozoite immune response in comparison with MAP bound to alum. The MAP bound to alum elicited high titers of antimimetic antibodies but hardly any sporozoite cross-reactive immune response. Unfortunately, no comparisons of MAP and PE construct, both in IRIV and on alum, are given.

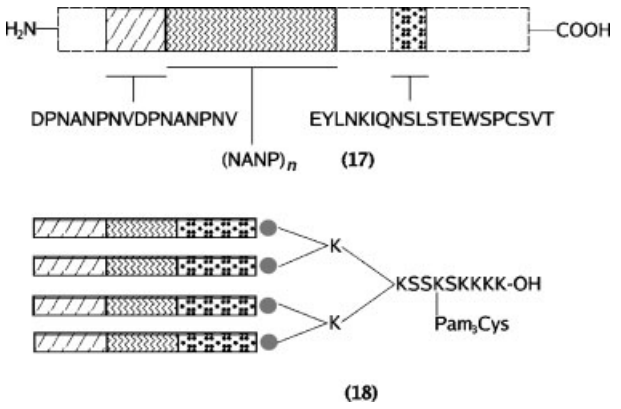


Figure 9 Illustration of *P. falciparum* CS protein with a T1 helper and B-cell epitopes within the central repeat region and the universal T * epitope in the *C*-terminus **17** and the lipopeptide P_3C-modified tetrabranched core **18** [131].

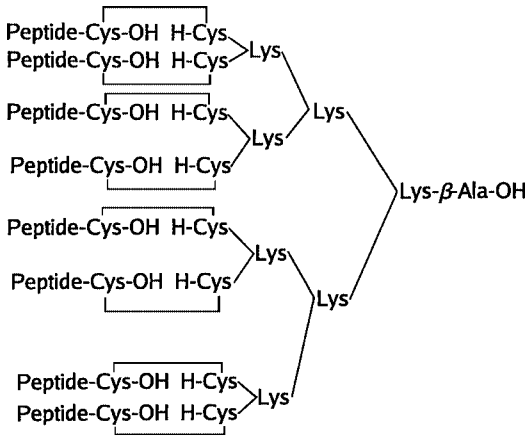
Avila *et al.* [132] evaluated the B- and T-cell response patterns in 66 individuals living in a malaria-endemic area of Brazil, a country where over 400 000 new cases of malaria occurred in 1998. Three types of MAPs $(T1B)_4$-MAP, B_4-MAP, $T1_4$-MAP, and two linear peptides T1B and B (T1 = DPNANPNVDPNANPV; B = NANPNANPNANP) have been prepared and tested. The highest frequencies of cellular responders have been obtained against $T1_4$-MAP and $(T1B)_4$-MAP (47 and 36%, respectively). The highest frequencies of humoral responders were obtained against $(T1B)_4$-MAP and B_4-MAP (42 and 29%, respectively). The results indicate that the ideal vaccine against *P. falciparum* malaria should be made up of multiple antigens containing B- and T-cell epitopes.

An effective malaria vaccine should contain a parasite-derived epitope to elicit $CD4^+$ T cells that could function as a source of both inhibitory lymphokines and T-helper factors for antibody responses. The authors [133] used a series of $CD4^+$ T-cell clones, derived from a volunteer immunized by irradiated *P. falciparum* sporozoites, to identify a class II restricted T-helper epitope, designated T1, in the 5′ repeat region of the CSP. This epitope consists of alternating NANP NVDP AAs sequences that are conserved in all *P. falciparum* strains. T1 is a 16-mer $(DPNANPNV)_2$ and B-cell epitope is a 12-mer $(NANP)_3$. The $(T1B)_4$-MAP $(DPNANPNVDPNANPNVNANPNANPNANP)_4$-$K_2$-K-Cys(Acm)-Ala-OH has been synthesized in a stepwise manner. This MAP has been assessed for safety and immunogenicity in volunteers of known class II genotypes. The MAP/alum/QS-21 vaccine formulation elicited high levels of parasite-specific antibodies in 10 of 12 volunteers expressing class II molecules. In contrast, volunteers of other HLA genotypes were low responders or nonresponders. This is the first demonstration in humans that a peptide vaccine containing minimal T- and B-cell epitopes composed of only five AAs (N, A, V, D and P) can elicit antibody titers comparable to multiple exposures to irradiated *P. falciparum* mosquitoes.

The heat-shock protein, Pf72/Hsp70-1 of the *P. falciparum* malaria parasite, is a major target molecule in the acquisition of immunity in naturally infected humans living in areas of endemic malaria. Analysis of the least conserved domain, the *C*-terminal domain, helped identify several B- and T-cell epitopes recognized in individuals living in an endemic area. One epitope, unique to *Plasmodium* species, was not identified

in human seroepidemiology studies, but has been specifically recognized by a MAb, 1C11. The authors [136] studied sera from humans living in areas of endemic malaria, who have been therefore sensitized as a result of *P. falciparum* infection and sera from animals immunized by injection with various formulations of the Pf72/Hsp70-1 antigen: the native antigen purified from the parasite, several recombinant proteins containing different regions of the *C*-terminal domain, monomeric synthetic peptides and MAP-P10 containing the 1C11 epitope. The MAP has been prepared from a prepurified Cys-containing peptide and $(Cys)_8$-Lys_4-Lys_2-Lys-β-Ala-OH by oxidation to give the desired MAP-P10 (Figure 10). Surprisingly, all human sera that reacted with the native antigen reacted strongly with the MAP-P10. This suggests that a B-epitope of the Pf72/Hsp70-1 antigen immunogenic in man has been mimicked in the MAP-P10, but not by the monomeric P10 peptide on enzyme-linked immunosorbent assay (ELISA) plates. The most interesting results concern the region present in peptide P10, which contains the epitope recognized by MAb, 1C11. The monomeric peptide P10 reacted strongly with the 1C11 MAb and with mouse sera, but it was not recognized by specific antibodies from monkeys immunized with the native or recombinant antigen. In contrast, the MAP-P10 reacted strongly with all sera tested, including those from individuals living in areas of endemic malaria.

It is known that the *P. yoelii* circumsporozoite protein AAs 57–70 region elicits T cells capable of eliminating infected hepatocytes *in vitro*. The authors [137] demonstrated that immunization of mice with a MAP $[(KIYNRNIVNRLLGD)_2$-$KGG]_2$-K-β-Ala-OH induced a peptide-specific, $CD8^+$-dependent, genetically restricted CTL response. The T cells resulting from the MAP immunization recognized naturally processed antigen as produced from infected cells, as demonstrated by their ability to eliminate infected hepatocytes



(19)

Figure 10 The structure of MAP-P10 **19**. Peptide = YGVKSSLEDQKIKEKLQPAEIETCGK [136].

in vitro, and therefore, the epitope has been classified as a subdominant epitope. Partial protection *in vivo* against sporozoite challenge has been also demonstrated. It is the first study in which an exact molecular nature of a *Plasmodium*-derived subdominant CTL epitope has been defined.

Peptide-based subunit vaccines against preerythrocytic stages of malaria parasites have been reviewed in general, including MAPs [171].

Antiviral Vaccines and Diagnostics

Human Immunodeficiency Virus (HIV). Owing to great variability of different pathogens (bacteria, viruses), new strategies to overcome the variability are needed. The conjugation of MAPs to carrier proteins can be one of the solutions [144]. Two peptides from the V3 loop of HIV-1 have been incorporated to the MAP as B-cell epitopes because of their variability. These MAPs have been conjugated to HBsAg. Groups of mice were immunized, and the immunogenicity and the level of cross-reaction to a panel of five heterologous V3 peptides have been studied. The results show that sera from mice immunized with MAP–HBsAg conjugate recognize a higher number of heterologous peptides.

The V3 loop epitope plays a critical role in HIV-1 neutralization and, therefore, this peptide is used in many HIV-1 vaccine candidates. In order to enhance cross-reactivity toward several V3 sequences, 50 peptides of the V3 region from HIV-1 subtype A have been used to create both a consensus peptide and a combinatorial peptide (mixotope) library representative of these sequences [145]. The consensus and mixotope immunogens have been incorporated into MAPs and conjugated to a recombinant surface antigen from HBsAg carrier protein and inoculated to mice together with a C4 (CD4 binding) MAP construction, also conjugated to HBsAg. Mice inoculated with the V3 consensus-MAP-HBsAg + C4-MAP-HBsAg mixture elicited higher antibody responses than those given the V3 mixotope-MAP-HBsAg + C4-MAP-HBsAg mixture. Besides, serum from the first group of immunogens had higher cross-reactivity against V3 peptides on cellulose membranes than those from mice given the combinatorial immunogen. The results suggest that the V3 consensus peptide is superior to the combinatorial strategy in inducing potent and cross-reactive responses to HIV.

The same authors [146] used different strategies to increase the immunogenicity of an antigenic HIV peptide as a vaccine candidate. The chosen B-cell epitope comprises 15 AAs (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 (TT). Different presentations including oligomerization, MAP dendrimers and conjugation to dextran beads have been synthesized and evaluated.

The MAP of the peptide conjugated to HBsAg protein has been a better immunogen than the MAP alone. It showed also higher immunogenicity than other multimeric presentations. The results are encouraging in the development of HIV vaccine candidates.

Cruz *et al.* [83] prepared one tetravalent and six octavalent MAPs bearing divergent V3 HIV-1 sequences LR150, JY1, RF, MN, BRVA and IIIB as B-cell epitopes and sequence 830–843 of (TT) as a T-helper cell epitope. All MAPs contained a 2 : 1 B-cell to T-cell epitope ratio. The MAPs have been prepared by oxidation of their smaller subunits and connected by S–S bonds of the Cys present in any subunit (Figure 11). The MAPs contained a cathepsin-like enzyme cleavage site KK between B- and T-cell epitopes. The structure of the tetravalent JY1-MAP4 was [(JY1-G)$_2$-KKK-TT epitope-GC-S]$_2$ and of the octavalent homodimeric JY1-MAP8 was {[(JY1-GG)$_2$-KKK-TT epitope]$_2$-KGC-S}$_2$. Heterodimeric MAPs containing 4 + 4 different epitopes have been prepared too. JY1-MAP8 elicited higher antibody titers in Balb/c mice than JY1-MAP4. This result is compatible with previous experiments in which octameric MAPs with higher B-epitope density have been more immunogenic. The results demonstrate that the heterodimeric V3-MAP8 mixture administered to mice induced a broader range of antibodies than the homodimeric variant. This suggests that V3 sequences presented in a heterodimeric MAP activate B-cell clones with a higher cross-reactive potential than those in the homodimeric context. These results emphasize the influence of V3 epitope presentation to the characteristics of the antibody response generated. A chimeric protein TAB 9 containing six copies of the central region of the V3 loop from HIV-1 isolates LR150, JY1, RF, MN, BRVA and IIIB has been prepared. A strong immunodominance towards JY1 and LR150 epitopes in mice immunized with TAB 9 was evident.

HIV-1 requires both CD4 and chemokine receptor for cellular entry. After the binding to CD4, the viral envelope protein changes its conformation to bind to the chemokine receptor and initiates fusion with the cellular membrane. CCR5 and CXCR4 are the main coreceptors for cellular entry of HIV-1. Therefore, viral strains are classified as R5, X4 or R5X4 according to the usage of chemokine receptor. HIV-1 R5 virus generally transmits the infection and predominates in the early stage of infection. HIV-1 X4 virus emerges during chronic infection, and its emergence is associated with a rapid decline in the count of CD4(+) T cells and progression to AIDS. CXCR4 is an attractive target that not only inhibits the entry of HIV-1 X4 virus but may also delay the disease progression to AIDS [147]. The authors investigated the application of a cyclic dodecapeptide (cDDX4) that was designed to mimic the native conformational epitope of the undecapeptidyl arch (UPA) in CXCR4 for use as a novel immunotherapy for AIDS. To mimic the conformational

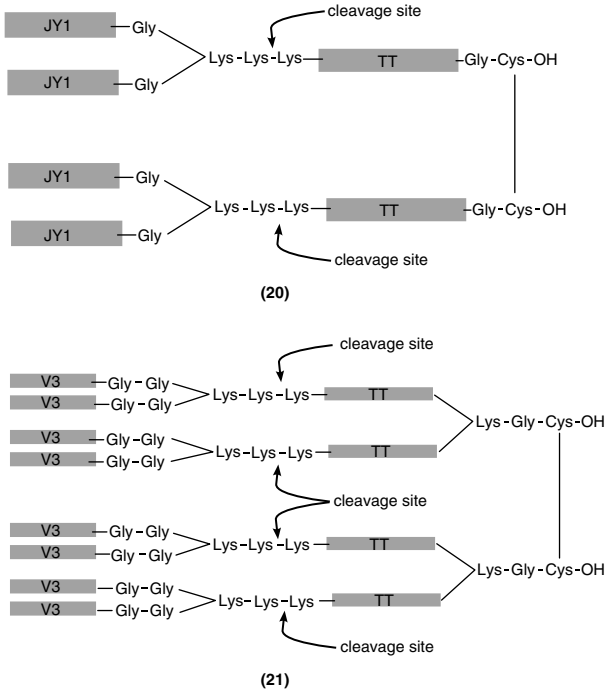


Figure 11 JY1-MAP4 **20** and V3-MAP8 **21** variants [83]. A cathepsin-like enzyme cleavage site (-KK-) was inserted between B- and T-cell epitopes.

epitope of human CXCR4, the CXCR4-derived linear dodecapeptide (linear DDX4: DNVSEADDRYIG) has been synthesized and cyclized. The obtained cyclic dodecapeptide (cDDX4) has been conjugated with the amino groups of MAP by the β-carboxyl group of Asp within the cDDX4. Immunization of BALB/c mice with cDDX4-MAP induced conformational epitope-specific antibodies and MAb LA2-F9 reacted with cDDX4, but not with linear DDX4. The conformational epitope-specific antibodies that preferentially recognized the cyclic structure of the antigen also reacted with cells expressing CXCR4 but not with cells expressing the other HIV coreceptor, CCR5. Furthermore, the antibody inhibited the replication of the HIV-1 X4 virus. The results indicate that cDDX4-MAP-induced conformational epitope-specific antibodies to the UPA of CXCR4 maybe a novel candidate immunogen for preventing disease progression in HIV-1 infected individuals.

The same group [148] worked up a similar way with the second main coreceptor for cellular entry of HIV-1, the CCR5. They used cyclic closed-chain dodecapeptide (cDDR5), which has been synthesized from linear DRSQKEGLHYTG. The peptide has been cyclized by bond formation between the COOH of Gly and α-amino group of Asp. The cyclic peptide was conjugated to the MAP by β-COOH of Asp. The resulting cDDR5-MAP, which mimics the conformation-critical domain for HIV-1 entry (the UPA, Arg$_{168}$ to Cys$_{178}$, of extracellular loop 2 (ECL2), was used as immunogen.

The cDDR5-MAP induced anti-CCR5 antibodies and inhibited infection by HIV-1 R5 in a dose-dependent manner, but did not suppress infection by HIV-1 X4.

Another paper of the Japanese group [149] deals with an analogous topic. Cyclic chimeric dodecapeptidyl multiple antigen peptide (cCD-MAP) has been constructed with a spacer-armed dipeptide Gly-Asp and two pentapeptides. The first has sequence 169–173 from CCR5 receptor, and the second one the sequence 179–183 from CXCR4 receptor. These receptors are components of the UPA of ECL2. ECL2 is shown to be very critical for the virus entry and signaling. The peptide DSQKEGEADDRG has been cyclized and conjugated to the MAP to give cyclic chimeric dodecapeptidyl MAP (cCD-MAP) as described above. Conformation-specific antibodies have been raised against cCD-MAP. One of the MAbs, CPMAb-I (IgMκ), reacted with cCD-MAP, but not with the linear chimeric dodecapeptide-MAP. The antibody reacted with the cells separately expressing CCR5 or CXCR4. The antibody markedly suppressed infection by X4, R5, or R5X4 virus in a dose-dependent manner.

The HIV envelope is composed of gp 120 and gp 41. The gp 120 allows virus binding to CD4(+) lymphocytes, whereas gp 41 mediates the subsequent virus/cell fusion that leads to HIV entry into the cell. During the events that trigger the fusion, the hypervariable V3 domain of gp 120 plays a key role through its interaction with lymphocyte surface coreceptors, e.g. CXCR4 and membrane antigens such as CD26 and proteases. MAP $(GPGRAF)_8$-K_4-K_2-K-β-Ala-OH is derived from the conserved GPGRAF sequence present at the tip of V3 loop of HIV envelope and has been prepared by SPPS. This MAP exerts a potent anti-HIV activity. The authors [150] identified the target mechanism of the MAP. Their results show that the MAP enters $CD4^+$ cells according to a receptor-mediated process. Thus, the MAP inhibits HIV infection likely through its interaction with CXCR4 as a part of its involvement in HIV entry into cells.

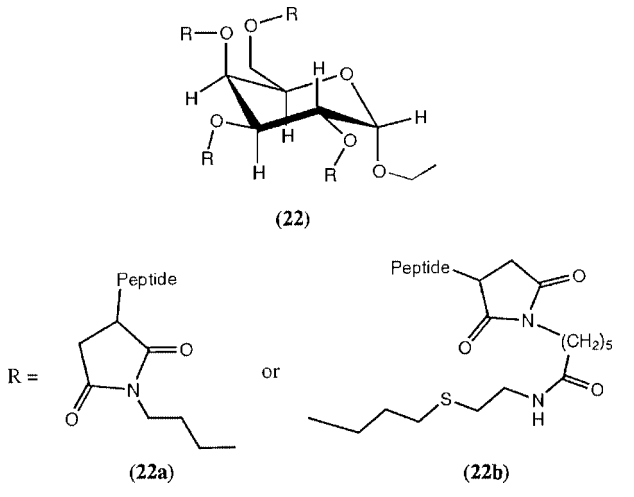


Figure 12 MAP **22** with carbohydrate core [153].

The synthesis of MAPs with a carbohydrate core, non-peptidic branches and peptide antigens on the surface has been described [153], (we put them under MAPs and not MAGs, because the antigen on the surface is a peptide). Carbohydrate core with branches containing maleimide was used as a new type of template for multivalent peptide assembly. Synthesis of two MAPs with two different branches was given (Figure 12). The peptide inhibitor T20 is a 36-mer peptide from the *C*-terminal ectodomain of HIV-1 gp41 with sequence Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWFC-NH_2. The two MAPs have been obtained by ligation chemistry, i.e. addition of the SH group from Cys of the peptide to the double bond of maleimide on the branches. The use of carbohydrates as the core molecule allows the topological display of multiple peptide chains with defined spatial orientations that depend on the nature of the sugar and the length of the spacer. The immunogenicity of the given MAPs is currently being evaluated.

The gp 160 precursor of HIV-1 envelope (Env) is processed into gp 120 and gp 41 by cellular subtilisin-like protein convertases. The gp 120 allows HIV attachment to target cells, mainly through binding to the primary CD4 receptor. The gp 160 cleavage occurs at the *C*-terminus of gp 120 within a cleavage region (gp120//PTKAKRR^{1}VVQREKR^{2}AVGIG//gp 41 for HIV_{Lai} Env) presenting two highly conserved endoproteolytic cleavage sites, named site^1 and site^2 [151]. A MAP $(PTKAKRRVVQREKR)_4$-K_2-K-β-Ala-OH that encompasses the cleavage region of the HIV-1 envelope precursor has been prepared. It displays an antiviral activity against HIV-1 and HIV-2 and inhibits HIV-1 Env-mediated cell-to-cell fusion. However, the MAP does not alter the status of Env cleavage at steady state. The MAP inhibits a step of the cell-to-cell fusion process after CD4 binding.

The same MAP $(PTKAKRRVVQREKR)_4$-K_2-K-β-Ala-OH containing an epitope from the *C*-terminus of the gp 120 subunit of HIV Env [151] was examined using a cell-to-cell fusion assay, receptor binding assays and molecular modeling to further address the characteristics of the peptide responsible for its anti-HIV activity [152]. The authors have shown that the MAP does not interfere with Env binding to CD4 and does not interact with the binding site of Env on CXCR4. The MAP does not inhibit protease activities. It interferes with processing of the gp 120 *C*-terminus at site^1 by the lymphocyte surface after CD4 binding. The authors propose that the cleavage region of Env is submitted to a stepwise processing including the known intracellular cleavage of gp 160 at site^2.

Diagnostics. Tandem repeats and MAPs have been compared as antigens to detect antibodies by enzyme immunoassay. The use of short peptides as antigens to achieve better specificity may jeopardize assay

sensitivity. A model system using V3-loop peptides derived from the HIV-1 subtype B consensus sequence has been presented [111]. In the given model, a tetravalent MAP was a better choice compared to the tandem repeats because of the MAPs higher sensitivity and lower cost of production.

Simian Immunodeficiency Virus (SIV). In Africa, there is a potential for additional cross-species transmissions from at least 33 different species of simian immunodeficiency virus (SIV)-infected nonhuman primates (NHPs) through hunting and butchering of these animals for food. A highly sensitive and specific enzyme-linked immunoassay (EIA) was developed [112] with MAPs for the detection and discrimination of antibodies to SIV genetic lineages. The SIV MAP EIA proved to be highly sensitive and specific for detecting SIV infections in NHPs and humans. This assay has the ability to detect previously unidentified SIV strains.

The stimulation of SIV_{mac}-specific antibodies and CTL in rhesus macaques using MAPs and P_3CSS-peptides (P_3CSS = tripalmitoyl-S-glyceryl-cysteinyl-seryl-seryl-) as immunogens has been studied [154]. MAP used for immunization of monkeys was R_8-Lys_4-Lys_2-Lys-β-Ala-OH (R = $gp130_{410-430}$ etc.) with known SIV_{mac} neutralization epitopes. Both humoral and CTL responses have been elicited. The peptide-specific antibodies induced by immunization could bind not only the relevant peptides but also virus proteins as demonstrated in ELISA and western blot. Although the V2 and V4 domains are known neutralizing epitopes, in this study they did not seem to play a major role in the neutralization of SIV_{mac}. None of the monkeys has been protected from infection but most demonstrated an anamnestic CTL response.

Equine Infectious Anemia Virus (EIAV). Equine infectious anemia virus (EIAV) is a member of the *Lentivirus* subfamily of retroviruses and possesses many of the characteristic features of the subfamily including a complex genome organization, tropism for cells of the monocyte/macrophage lineage and establishment of a persistent lifelong infection. EIAV is naturally transmitted by insects that bite such as horseflies that transfer blood from viremic horses to susceptible horses. Following infection, the virus replicates in terminally differentiated tissue macrophages and establishes a cell free viremia. One feature that distinguishes EIAV from other lentiviruses is the nature of clinical disease. Whereas many lentivirus infections are characterized by a slow, chronic, progressive disease course, EIAV infection results in a rapid, variable and dynamic disease course. Moreover, horses that survive the early clinical episodes of the disease are generally able to control virus replication even in the face of antigenic variation and remain clinically normal, unapparent carriers of EIAV. The authors [155] studied the immunogenicity of EIAV Gag and Env equine leukocyte alloantigen (ELA)-A5.1, -A9 and -A1 restricted (CTL) epitopes synthesized in MAP format. The MAP was coupled to tripalmitoyl-S-glycerylcysteine (P_3C) (Figure 13). The P_3C-MAP stimulated peripheral blood mononuclear cells (PBMCs) from horses chronically infected with EIAV. The stimulated CTL lysed EIAV-infected target cells, and the percent specific lysis was dependent on the dose of P_3C-MAP used to stimulate PBMCs *in vitro* and has been peptide specific and ELA-A restricted. The P_3C-MAP was far better than free peptides or virus in stimulating CTLm from some horses. The P_3C-MAP can be used as an immunogen to stimulate primary immune responses *in vivo*.

Influenza vaccines. Current influenza virus vaccines aim at inducing a strong Ab response to two transmembrane proteins which are expressed in the membrane of influenza type A virions and virus-infected cells. They are the HA and neuraminidase (NA), glycoproteins with large ectodomains of 510 and 420 AAs, respectively. The antivirus vaccines targeted at HA or NA are problematic, owing to great variability of these structures. To overcome this problem, the authors [158] focused on matrix protein 2 (M2), which contains ectodomain (M2e) that is highly conserved among human influenza virus strains. M2e is 23 AAs long. MAP construct containing M2e and linked helper T-cell determinants has been prepared. Mice inoculated twice by the intranasal route with MAPs containing multiple M2e peptides and Th determinants exhibited significant resistance against subsequent challenges with infectious virus. The authors have used the term MAP not in the classical sense, but we have included it here because it is a branched structure containing Lys; see Figure 14.

Hepatitis vaccines. Immunogenetic properties of a tetrameric heterogeneous palmitoyl-derived MAP containing two defined HAV (hepatitis A virus) peptide sequences, VP1(AAs 11–25) and VP3(AAs 102–121), in rabbits immunized with either Freunds complete

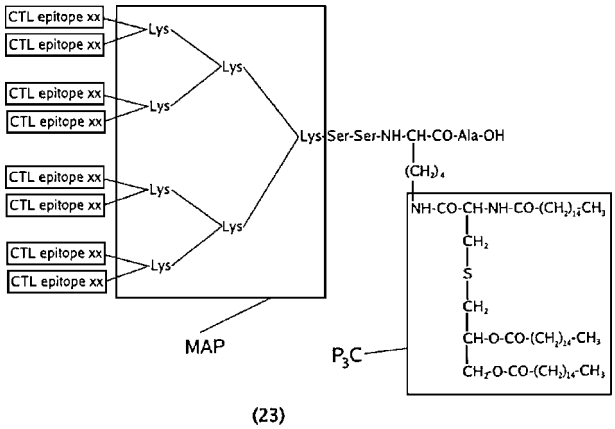


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Figure 13 Structure of P_3C-MAP with eight identical peptides **23**. The 'xx' indicates carboxy-terminal amino acids from the Gag p26 or Env SU protein [155].

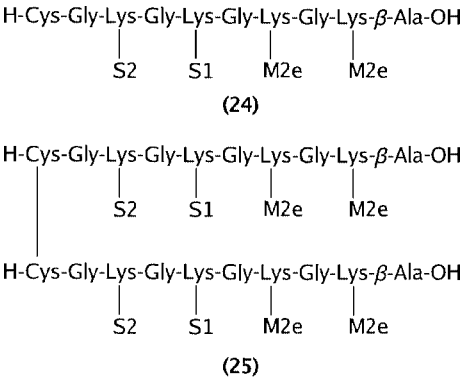

Figure 14 Some peptides (**24,25**) used for antibody production [158]. M2e = SLLTEVETPIRNEWGCRSNDSSDP; S1 = SFERFEIFPKE; S2 = HNTNGVTAASSHE.

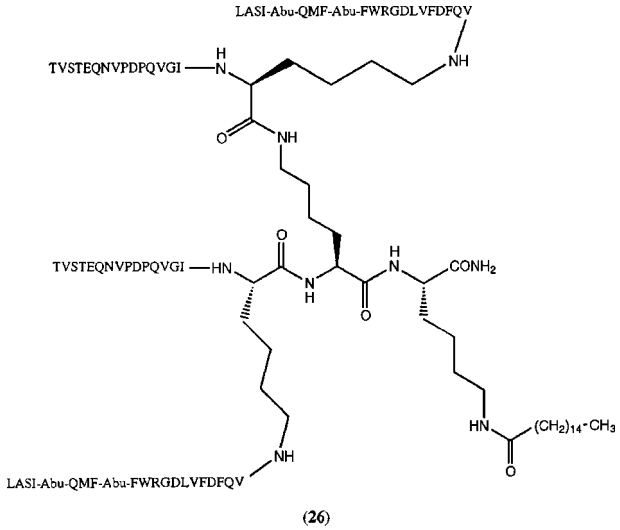

Figure 15 Primary structure of [VP1 + VP3]MAP **26** [159].

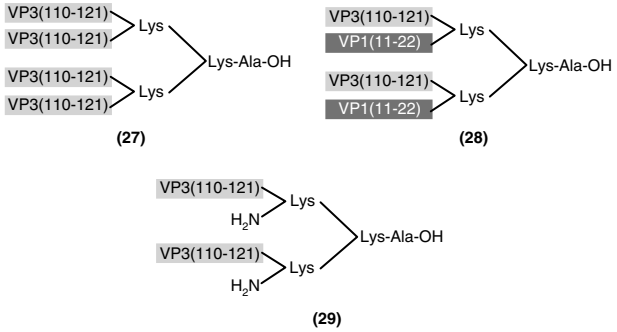

Figure 16 Sequences of linear and branched synthetic peptides **27**, **28** and **29** [104].

adjuvant (FCA) or multilamellar vesicle (MLV) liposomes were studied. For formulation of an effective immunogenic preparation, MLV liposomes have been prepared in order to achieve good yields in the MAP incorporation process. The palmitoyl moiety of tetravalent bis-diepitopic [VP1 + VP3]MAP (Figure 15) modifies the hydrophobicity of the MAP and favors the incorporation into the liposome. The rationale for anchoring the palmitoyl-[VP1 + VP3]MAP into the liposomes was to mimic the external appearance of the virion, particularly of the surface protein. The authors [159] observed that in rabbit No. 2 immunization with palmitoyl-[VP1 + VP3]MAP in liposomes elicited specific IgG not only against the MAP itself but also against the VP3 and VP1 antigens alone. However, in rabbit No. 1 (immunized with palmitoyl-[VP1 + VP3]MAP and FCA) no recognition was observed for the V3 peptide. In the light of these results, the MAP constructs are considered suitable to be used as immunogens mimicking the native protein, and the liposome formulation of the lipophilic MAP offers a valuable strategy for the design of therapeutic vaccines.

A random peptide library has been used to acquire the phage-displayed mimotopes that mimic the specificity of HBsAg. Two phagotopes have been identified and one of them was confirmed as a mimotope by competition experiment [160]. Octavalent MAP (MAP 1) containing the mimotope TANGFYRLPSGS and control MAP (MAP 2) based on the epitope of AAs 130–150 of HBsAg have been prepared by SPPS. BALB/c mice have been immunized i.p. with MAP 1 or MAP 2 in FCA. The results showed that the mimotope-based antigen (MAP 1) evoked higher titer of antibodies with the same specificity of the epitope-based antigen. Therefore, mimotopes can be used in antigen and vaccine design.

Hepatitis serodiagnosis. Researchers [104] have investigated the application of a commercial biosensor instrument (BIACORE 1000, Biacore AB) for the detection of antibodies against the HAV in human serum samples. Four synthetic constructs (one linear peptide and three MAPs) have been prepared: linear peptide VP3 (110–121; FWRGDLVFDFQV) from capside protein HAV, $(VP3)_4$-MAP **27**, $(VP3)_2(VP1)_2$-MAP **28** and $(VP3)_2$-MAP **29** (Figure 16). Peptide constructs have been covalently coupled to the sensor chip in order to ascertain their ability to detect anti-HAV antibodies in a panel of 25 positive sera. Linear VP3 recognized 22 of 25 positive sera samples (sensitivity 88%). When the $(VP3)_2$-MAP **29** was used, the sensitivity increased to 96% (24 of 25 positive sera samples). The reactivity observed with $(VP3)_4$-MAP and $(VP3)_2(VP1)_2$-MAP was significantly lower, 12 from 25 positive sera and 15 from 25 sera, respectively. The linear VP3 and divalent $(VP3)_2$-MAP have been the most sensitive and appropriate for serological studies of sera from HAV-infected patients using BIACORE. One explanation for the drop of reactivity with $(VP3)_4$-MAP and $(VP3)_2(VP1)_2$-MAP could be that the steric hindrance interfered with antibody recognition. Another possibility could be the difficult immobilization process by means of covalent linkage of the MAP. The CD analysis supported the tendency of tetravalent MAPs to adopt β-sheet structures

because of intramolecular aggregation, thus limiting the epitope accessibility.

A fluorescence study on the interaction of a multiple antigenic peptide from hepatitis A virus with lipid vesicles has been performed [105] using $(VP3)_4$-MAP. Small unilamellar vesicles of different compositions, including zwitterionic dipalmitoylphosphatidylcholine (DPPC), anionic dipalmitoylphosphatidylcholine/phosphatidylinositol (DPPC : PI 9 : 1) and cationic dipalmitoylphosphatidylcholine/stearylamine (DPPC : SA 9.5 : 0.5) have been used as membrane models. The $(VP3)_4$-MAP binds to all three types of vesicles with the same stoichiometry. The MAP interacts strongly with the liquid components of the membrane, and although binding is not of electrostatic nature, the bound form of the peptide has a different activity depending on the membrane net charge; thus, it is membrane-disruptive in cationic and anionic vesicles, whereas no destabilizing effect is seen in DPPC vesicles.

In an attempt to develop a synthetic peptide–based ELISA that can be used as a screening test for acute HAV infection, the recognition of different peptide constructs (including MAPs) from VP1, VP2 and VP3 HAV capside proteins by two panels of serum samples from two distinct populations (Chile and Spain) has been studied [106]. For this purpose $(VP1)_4$-MAP, $(VP2)_4$-MAP, $(VP3)_4$-MAP, $(VP3)_2(VP1)_2$-MAP and $(VP3)_2$-MAP and lipophilic, palmitoylated Palm-VP3, Palm-VP3(102–121) and $(Palm\text{-}VP1)_2$-MAP, where VP1(11–25) = TVSTEQNVPDPQVGI, VP2(96–107) = GLLRYHTYARFG, VP3(110–121) = FWRGDLVFDFQV and VP3(102–121) = ASICQMFCFWRGDLVFDFQV (see also Ref. 104 and Figure 16) have been prepared and tested. A patient serum recognized preferentially homogeneous double branched $(VP3(110–121))_2$-MAP and Palm-VP3(110–121). The Palm-VP3(110–121) and the dimeric $(VP3(110–121))_2$-MAP have been the most sensitive and appropriate for serological studies of HAV-infected patients by ELISA, sensitivity and specificity being higher than 90 and 95% respectively.

Epstein–Barr Virus (EBV) vaccine. MAbs essential for the development of assay systems, particularly where antigens have been developed using synthetic peptides, have been characterized. For this purpose, MAPs have been used for immunization [122]. BALB/c mice have been immunized by three MAPs of Epstein–Barr virus (EBV) latent membrane protein 1 (LMP1). The polyclonal antibody (PAb) responses showed that MAPs evoked B-cell responses. However, on screening 144 hybridomas, 24 MAb supernatants exhibited weak to moderate reactivity in ELISA and against cell cytospin preparations B95.8 and AG876 LCL, respectively. Characterization of hybridoma supernatants showed that 11 out of 24 have been IgM. It is probable that these MAPs failed to augment T-cell help and contributed to the production of low affinity IgM antibodies.

Infectious Bursal Disease Virus (IBDV). MAPs related to antigenic determinants of infectious bursal disease virus (IBDV) have been used for detection of an anti-IBDV-specific antibody in ELISA [109]. MAPs have been prepared from the predicted antigenic determinants on the VP2 protein of IBDV and used as antigens in ELISA, an alternative to whole viral antigen to detect anti-IBDV antibodies in the chicken sera. The two MAPs synthesized could specifically detect the anti-IBDV antibodies in serum samples by ELISA. The optimum amount of MAP1 and MAP2 required for ELISA was 5 ng/ml. The amount of purified IBDV whole viral antigen was 500 ng/ml. This indicates the high efficiency of both MAPs. The octabranched MAPs can serve as safe, chemically defined, noninfectious alternative antigens to the whole virus in serodiagnosis.

Herpes Simplex Virus (HSV). Herpes simplex virus type-2 (HSV-2) is the main causative agent of recurrent genital herpes. The majority of patients with HSV-2 antibodies do not realize that they are infected with the virus since they are either asymptomatic or have lesions localized in areas such as the cervix which are difficult to observe. A further large percentage of patients with symptoms do not recognize that they are infected since neither they nor their physicians recognize these as being caused by HSV. Primary infection during pregnancy has been associated with spontaneous abortion, prematurity and congenial infection, while both primary and recurrent genital herpes may result in neonatal herpes. In addition, HSV-2 is known to cause breaks in the genital mucosal barrier thereby increasing the risk of acquisition and transmission of HIV. Therefore, the diagnostics of HSV-2 is of utmost importance. The HSV-2 type-specific glycoprotein G2 (gG2) is the most acceptable and widely used type-specific antigen for serological diagnosis of HSV-2 infections. The authors [113] compared this antigen, gG2, to a previously recognized immunodominant epitope spanning residues 561–578 of the gG2 named peptide 55 (PEEFEGAGDGEPPEDDDS) directly for type-specific serodiagnosis of HSV-2. The immunodominant epitope (peptide 55) has been used as tetravalent MAP with four Gly as a linker, i.e. $(PEEFEGAGDGEPPEDDDSGGGG)_4$-$K_2$-K-Ala-OH. A panel of 100 characterized serum samples (60 HSV-2 positive, 20 HSV-1 positive and 20 HSV negative) was screened using G2 and MAP as antigens, respectively. The MAP and native immunoaffinity purified gG2 showed the same sensitivity for the detection of antibodies in the serum of HSV-2 infected individuals, reacting with 58 from 60 positive samples (sensitivity 96.7%). The MAP did not react with any of the HSV-1 positive or HSV negative sera. In contrast, gG2 gave a number of false positive results, reacting with 20% of the HSV-1 positive sera and 10% of HSV negative sera. Another advantage of the MAP over gG2 is

100–300 times lower cost of production per one assay. The MAP derived from peptide 55 should become the antigen of choice in enzyme immunoassays for type-specific serodiagnosis of HSV-2 infections.

The strength and specificity of antibody binding could be altered by conjugation to macromolecules or by modification in the flanking regions. The influence of carrier structure and topology on antibody recognition of an attached epitope has been studied by comparing the antibody-binding properties of a new set of conjugates with tetratuftsin analogue H-(Thr-Lys-Pro-Lys-Gly)$_4$-NH_2 (T20), sequential oligopeptide carrier $(SOC)_n$, branched chain polypeptide poly[Lys(Ser$_i$-DL-Ala$_m$)] (SAK), MAP and keyhole limpet hemocyanine (KLH) [114]. Peptide LKNleADPNRFRGKDL ([Nle^{11}]-9-22) representing an immunodominant B-cell epitope of herpes simplex virus type 1 glycoprotein D (HSV-1 gD) has been conjugated to the above-mentioned structures via a thioether or amide bond. Direct and competitive ELISA with MAb A 16, recognizing the HSV gD-related epitope [Nle^{11}]-9-22 and the five conjugates containing an identical and uniformly oriented epitope peptide, have been compared. The results of the MAb binding experiments indicated that the carrier-bound peptide epitopes are recognized more efficiently than free peptides in all multivalent peptide conjugates except MAP and KLH conjugate. It is important to stress that these conclusions are valid only for the given model and epitope and in other cases the results could be different.

Human Herpes Virus (HHV). The mapping of the immunodominant region of the HHV-8, the antibody-binding site of glycoprotein K8.1A, has been carried out by using overlapping peptides and a residue replacement method [115]. The main epitope was found within residues 44–56. Tetravalent MAP has been used as an antigen to develop an enzyme immunoassay to detect HHV-8 antibodies in human sera. The sensitivity and specificity of the assay have been 96 and 99.4%, respectively. The assay should be useful for population-based, epidemiological studies of HHV-8 infection.

Measles Virus (MV). The development and use of potent adjuvants for human mucosally delivered vaccines is limited by toxicity. Novel adjuvant formulations have been used for intranasal immunization with an MAP-M2 [156]. The peptide mimic (M2) of a conformational B-cell epitope from measles F protein with sequence NIIRTKKQ has been incorporated into octavalent MAP. Toxin LT of *E. coli* has been used throughout this study as a positive control, since, when coimmunized with MAP-M2, it induced high serum antibody titers and a large number of antibody-secreting cells in bone marrow. Similar results have been obtained when the mutant of LT, LTR72, has been used as an adjuvant. Synthetic oligodeoxynucleotides that contain unmethylated CpG motifs (CpG) are also novel candidates as adjuvants. The combination of the mutant toxin LTR72 and the CpG repeats codelivered with the MAP-M2 induced local and systemic peptide- and pathogen-specific humoral and cellular immune responses is comparable to those obtained after intranasal immunization with the wild-type toxin LT. If both the LTR72 and CpG adjuvants are shown to be safe for use in humans, this combination would appear to have potential as an adjuvant for mucosally delivered vaccines in humans.

The same authors [157] compared MAP-M2 with a chimeric peptide consisting of two copies of a T-helper epitope (residues 288–302 of MV fusion protein) and one copy of the mimotope M2. The octavalent MAP-M2 induced the highest titers of anti-M2 and anti-MV antibodies. Immunization with MAP-M2 construct induced high titers of a high-affinity anti-M2 antibody despite the absence of a T-helper epitope.

Nasal delivery of epitope-based vaccines, including MAPs, has been reviewed [172].

Antibacterial Vaccines

Mycobacterium tuberculosis. More than 80 million new cases of tuberculosis are expected in the coming decade and an increasing proportion of these are likely to result from drug-resistant strains of *Mycobacterium tuberculosis*. The only vaccine currently available against *M. tuberculosis*, *Mycobacterium bovis* BCG, has shown variable protective efficacy ranging from 0 to 85% in different field trials. Therefore, a new generation of vaccines is urgently needed. The use of synthetic peptides for vaccination represents an attractive alternative because of its simplicity and safety. The 6 kDa early secretory antigenic target (ESAT) of *M. tuberculosis* is strongly recognized by Th1 cells in the early phase of infection in patients as well as in experimental animals. The authors [164] evaluated the potential of peptide-based vaccines and investigated the relationship between the hierarchy of epitopes recognized during infection and their vaccine potential. ESAT-6_{1-20} (MTEQQWNFAGIEAAASAIQG), ESAT-6_{51-70} (YQGVQQKWDATATELNNALQ) and the corresponding MAPs (ESAT-$6_{1-20})_4$-MAP and (ESAT-$6_{51-70})_4$-MAP have been tested for their ability to induce a systemic immune response and protection against tuberculosis infection. All four tested compounds have been highly immunogenic and have induced cellular responses of the same magnitude. Compared to the MAP constructs, immunization with the free peptides induced lower but similar recall response. There is no direct correlation between the hierarchy of response to naturally processed peptides and their ability to induce protective immunity against *M. tuberculosis.*

Pneumococcal capsular polysaccharide. Peptide mimotopes as prototypic templates of broad spectrum surrogates of carbohydrate antigens have been studied

[165]. A peptide mimetic (peptide 105) of the pneumococcal capsular polysaccharide type 14 (Pn14) has been studied as a model antigen to explore differences in antigenicity and immunogenicity of peptide mimotopes. The corresponding MAP competes in ELISA with native Pn14 in a concentration-dependent manner for binding to an anti-Pn14 MAb. Such peptides would simplify currently available vaccine approaches.

Streptococcus mutans. *Streptococcus mutans* contains glucan-binding protein B (GbpB). It has been shown that this protein induces protective immunity to dental caries in an experimental model. The objective has been to identify immunogenic regions within the GbpB sequence suitable for use in subunit vaccines. The regions of immunogenicity have been sought by use of a matrix-based algorithm (EpiMatrix) to estimate the binding characteristics of peptides derived from the GbpB sequence by using a database of known major histocompatibility complex MHC class II binding alleles. The screening of the entire sequence revealed several promising peptides with high binding probabilities [166]. Two *N*-terminal peptides (SYI and QGQ) subtending two of these regions have been synthesized. Peptides SYI (KSNAATSYINAIINSKSVSD; GbpB residues 113–132) and QGQ (KHKLITIQGQVSALQTQQAG; GbpB residues 57–71; residues KHKLI are irrelevant to GbpB sequence) have been prepared in the form of tetravalent MAPs. The MAPs have been used to subcutaneously immunize Sprague–Dawley rats. The SYI-MAP induced a higher percentage of responses to the inciting peptide as well as to intact GbpB, as measured by ELISA. The effect of immunization with the SYI-MAP on the cariogenicity of *S. mutans* has been also investigated on weanling Sprague–Dawley rats. All rats have been then orally infected with the *S. mutans* strain SJ. After a 78-day infection period, the SYI-MAP-immunized animals had significant reductions in dental caries on both smooth and occlusal surfaces. The results show that at least one linear sequence derived from the *N*-terminal third of GbpB has been able to induce protective immune responses in the given model for dental caries. These results should enhance the effectiveness of subunit-based dental vaccines.

The same group [167] explored the immune potential of a tetravalent MAP construct combining epitopes from mutans streptococcal GTF and GbpB. The origin of dental caries is dependent on the ability of *Streptococci mutans* to accumulate on dental surfaces. Both GTF and Gbp are essential to the pathogenicity of *Streptococci*. Two diepitopic tetravalent MAPs have been synthesized. Both MAPs contained SYI peptides, a 20-mer GbpB peptide that was selected on the basis of MHC class II binding characteristics and the ability to induce caries-protective activity after immunization. These SYI sequences have been combined with either CAT- or GLU-peptides to form $(SYI)_2(CAT)_2$-MAP or $(SYI)_2(GLU)_2$-MAP. CAT is a 22 AAs sequence from the catalytic domain of GTF and GLU is a 22 AAs sequence from the glucan-binding domain of GTF. The rats were subcutaneously injected with $(SYI)_2(CAT)_2$-MAP or $(SYI)_2(GLU)_2$-MAP, followed by infection with *S. mutans* and *Streptococcus sobrinus*. Dental caries have been lower in each peptide-immunized group than in the sham-injected group but $(SYI)_2(CAT)_2$-MAP enhanced both the immunological response to CAT and GTF epitopes and extended the protective effect to *S. mutans* and *S. sobrinus.*

Group A Streptococci (GAS). *Group A streptococci* are human pathogens that cause a variety of illnesses and diseases, ranging from the relatively minor pharyngitis to more severe invasive diseases, and the poststreptococcal sequelae-rheumatic heart disease and acute glomerulonephritis. The most important virulence factor is the M-protein. The LCP [85] containing four copies of peptide J8 from the conserved region of the M-protein has been prepared by SPPS. The sequence of J8 is QAEDKVKQSREAKKQVEKALKQLEDKVQ. For the structure of LCP (MAP) see Figure 5, compound **13**. The LCP has been lipophilized by three consecutive 2-amino-dodecanoic acids with glycine spacers. The LCP construct or the J8 peptide has been used to immunize mice by the parenteral route with and without FCA. J8-specific antibodies have been detected in all mice immunized with LCP in FCA and J8 in FCA, giving a final average antibody titer after five boosts of 1 365 000 and 747 500, respectively. No J8-specific antibodies have been detected in mice immunized with J8 without adjuvants. J8-specific antibodies have been not detected 3 weeks after immunization with LCP without adjuvants. After the final boost 5, the average J8 antibody titer in mice immunized with the LCP construct without adjuvant was 49 500.

Antifungal Vaccines

Paracoccidioidomycosis (PCM). The major diagnostic antigen of PCM, a prevalent fungal infection in South America, is the 43-kDa glycoprotein (gp43) of *Paracoccidioides brasilliensis*. A MAP with the protective T-cell epitope expressed in a tetravalent 13-mer sequence from gp43 has been prepared (M10). M10 induced specific lymph node cell proliferation in mice preimmunized with peptides in FCA. Besides, M10 immunization without FCA significantly protected intratracheally infected mice. The authors conclude that M10 is a candidate for an anti-PCM vaccine [163].

Antiprion antibodies. Prion diseases are rare neurodegenerative disorders in which endogenous glycoprotein, termed *cellular prion protein* (PrP^c), mainly expressed in the CNS and lymph tissues, is refolded to an altered pathogenic isoform enriched with β-sheet structures termed *scrapie prion protein* (PrP^{Sc}). All forms

of prion diseases share common neurodegenerative histopathologies, resulting in spongiform-like brain tissue, neuronal loss, astrocytic gliosis and accumulation of abnormal PrP^{Sc} in the brain and spleen, which in some cases forms amyloid fibrils. Owing to the relatively poor immunogenicity of both PrP^{c} and PrP^{Sc}, the generation of antiprion antibodies causes significant problems in the development of immunotherapeutic strategies. Therefore, MAPs have been studied in order to raise antibody response to prion-derived sequences in mice [161]. The MAP was constructed of a four-spiked ring. Two spikes contained human- or mouse-derived prion AA sequences and two spikes contained the universally promiscuous TT sequence (AAs 830–844), which has been used to support T-cell dependent B-cell antibody production. When the MAP contained only the mouse sequence, it failed to elicit a significant antibody response. MAPs with human prion sequences elicited antibody production to the corresponding prion sequence. These peptides have been able to generate antibody responses recognizing conserved human and mouse sequences. These homologous sequences contain the heralded PrP^{Sc} specific sequence Tyr-Tyr-Arg and therefore may have some therapeutic potential.

The difficulty in obtaining PrP-reactive antibodies has been overcome by using octavalent MAP [162]. A fragment of PrP helix 1 (AAs 144–153 of human PrP) DYEDRYYREN was bound to the octavalent MAP. A high titer of IgG antibodies against MAP-helix 1 has been obtained in BALB/C mice after three immunizations with MAP-helix 1 associated with FCA, which reached a maximal end point titer of 1 : 1 000 000. It is important that purified IgG binds the whole prion protein with high affinity. MAPs containing different PrP peptides can be used to elicit anti-PrP antibodies for diagnostic purposes or even to generate PrP^{Sc}-specific antibodies.

Alzheimer's disease. Complement C5a receptor-mediated signaling, which can be involved in neurodegeneration in Alzheimer's disease has been studied using octavalent MAPs [116].

Allergens. MAP of the IgE-reactive linear epitope 3 (AAs positions 27–36) Ara h 2 (the major peanut allergen) has been prepared [108]. The MAP has been used to immunize rabbits (with Titermax TM as adjuvant) to raise high titer antibodies and to characterize the specificity of IgE from allergenic patients sensitized to Ara h 2. The antiserum selectively detects Ara h 2 in crude peanut extract with a titer of 10^{-7} by western blot and reacts specifically with epitope 3. These IgE-reactive epitopes are of high analytical relevance because they could constitute the basis for epitope-specific detection systems for quality control in the food industry and for forensic purposes in cases of fatal reactions to otherwise undetected peanut proteins.

Autoimmune Diseases

Experimental Autoimmune Encephalomyelitis (EAE). EAE is an induced disease that affects the CNS and serves as a model for human multiple sclerosis. The induction of EAE can be done by intraperitoneal or subcutaneous injection of oligodendrocyte membrane proteins, i.e. myelin basic protein, proteolipid protein or myelin oligodendrocyte glycoprotein (MOG). The MOG protein contains three main regions. The authors [117] describe an efficient immunization protocol for C57BL6/J mice, using MAP containing eight $pMOG_{35-55}$ (fragments of extracellular MOG domain). The structure of $pMOG_{35-55}$-MAP was $(pMOG_{35-55})_8$-K_4-K_2-K-β-Ala-OH. The AA sequence of $pMOG_{35-55}$ is MEVGWYRSPFSRVVHLYRNGK. The $pMOG_{35-55}$-MAP has been highly immunogenic and has induced severe clinical symptoms even in the absence of the *Bordetella pertussis* toxin. Using this protocol, 50% of the mice had primary-progressive disease, while the other 50% had a chronic relapse-remitting EAE.

Systemic Lupus Erythematosus (SLE). SLE is an autoimmune rheumatic disease serologically characterized by production of a variety of autoantibodies. Antibodies to double-stranded (ds) DNA are considered to be a diagnostic marker in SLE. Their presence often correlates with an active disease [118]. It was found that the murine R4A anti/dsDNA antibodies cross-react with R4A peptide. While monomeric peptide has been unable to inhibit affinity-purified polyclonal anti-DNA antibodies, serum anti-DNA reactivity has been inhibited by an octavalent $(DWEYSVWSLN)_8$-MAP in 10 SLE patients. The MAPs might be a useful surrogate marker for SLE. Anti-β2glycoprotein I (anti-β2GPI) antibodies constitute the main autoantibody specificity in the sera of patients with antiphospholipid syndrome (APS). APS is an acquired, immune-mediated thrombophilia, defined as a combination of thrombosis or pregnancy morbidity with antibodies to phospholipids and, more specifically, anticardiolipin antibodies or lupus anticoagulant. The anti-β2GPI antibodies induce the precoagulant activity of the endothelium by cross-linking the β2GPI on the cell surface. Since β2GPI lacks intracellular domains, homology with other molecules such as CD40 that could initiate signaling has been searched [119]. An 86% homology between the AA position 239–245 of the CD40 and 7–13 of the β2glycoprotein has been found. A MAP containing the peptide FPDDLPGSN corresponding to the AA positions 239–245 of the human CD40 extended by 4 AAs at its aminoterminal and by 1 AA at its carboxy-terminal has been synthesized. The $(QEINFPDDLPGSNT)_4$-MAP (CD40 MAP) has been used as the antigen in an ELISA to detect anti-CD40 MAP reactivity in the sera of APS patients and controls. The CD40 MAP has been found to preferentially react with sera of patients with APS and SLE, but not in patients with rheumatoid arthritis or normal sera.

Gerli *et al.* [120] investigated the association of ribosomal anti-P antibodies (anti-P) with serological findings and clinical manifestations including neuropsychiatric involvement in a large group of patients with SLE. Anti-P have been evaluated in the serum of 149 consecutive Italian SLE patients by an ELISA using MAPs. The MAPs carried four copies of a common P0, P1 or P2 epitope. Serum anti-P have been detected in 18/149 patients (12.1%). A strong association between IgG anticardiolipin antibodies and anti-P has been also found. The study does not confirm the described association of anti-P with SLE neuropsychiatric manifestations.

The same group of authors [121] used tetravalent MAPs containing four copies of the *C*-terminal 13 AAs long P-peptide for diagnostic tests for antiribosomal P-protein antibodies.

Contraception Vaccines

The PH-20 protein is localized on the sperm surface and its multiple functions during fertilization are well known. Therefore, it is a potential target for contraceptive vaccines [138]. Cynomolgus macaques have been immunized using four adjuvants together with synthesized peptides or recombinant proteins representing selected regions of macaque PH-20 protein. The synthesized peptide (AAs 387–412, designated Peptide 4) has been used as MAP construct. The circulating antibodies from immunized animals recognized the macaque sperm surface PH-20 on western blots and were shown by indirect immunofluorescence to bind to the surface of macaque sperm. From the adjuvants used, Montanide and Titermax were associated with higher titers of antibodies than QS-21 and SAF adjuvants. MAPs and recombinant proteins representing selected regions of the PH-20 molecule can be used as vaccine components in combination with the adjuvant Montanide to elicit a significant sperm-directed antibody response in immunized macaques.

Antitumor Vaccines

Human malignant gliomas contain epidermal growth factor receptor (EGFR) gene mutations that encode tumor-associated antigens (TAAs). They can be targeted by immunological techniques. One EGFR mutant gene (EGFRvIII) encodes a protein with an epitope not found in normal tissues. This study [140] focused to the cellular immune response of MAP containing multiple copies of the unique EGFRvIII epitope. The median survival of Fischer rats vaccinated with the above-mentioned MAP was increased 72% over that of unvaccinated controls challenged with intracerebral F98EGFRvIII tumor implants.

Analysis of CD8 T-cell response by IFNγ ELISPOT and H-2L(d)/pRL1a tetramer assays in pRL1a multiple antigen peptide-immunized and RL male 1-bearing BALB/c and (BALB/cxC57BL/6) F-1 mice has been studied using octavalent MAPs [139].

T-cell recognized antigens for which no molecular information is available, have been identified [141]. A unique tumor antigen peptide pRL1a, IPGLPLSL that is recognized by CTL on BALB/c RL male 1 leukemia has been identified by peptide elution. Cytotoxicity has been generated in BALB/c spleen cells by *in vivo* and *in vitro* sensitization with pRL1a peptide in the form of MAP, but not in the original form. Immunization with pRL1a MAP had a significant growth-inhibitory effect.

Cellular processing of a multibranched lysine core (MAP) with tumor antigen peptides and presentation of peptide epitopes recognized by CTL on antigen-presenting cells has been studied [81]. It is generally known that tumor antigen peptides are weakly immunogenic. An antitumor vaccine must elicit a protective immune response. The antigen peptide pRL1a, present on a murine leukemia RL male 1 has been incorporated into the MAP, $(IPGLPLSL)_8$-K_4-K_2-K-β-Ala-OH. The MAP was chosen in order to increase the immunogenicity. Immunization with the MAP generated efficiently a specific CTL response in BALB/c mice and protected the mice from RL male 1 tumor growth. The study showed the processing of MAP in the MHC class 1 pathway and elucidated the basis for the effect of MAP as a tumor vaccine.

The ability of some tumor cells to create metastasis is associated with the expression of the neolactoseries antigens sialyl-Lewis x (sLex) and sialyl-Lewis a (sLea) as they are ligands for selectins. L-selectin expressed on lymphocytes has also been noted in that anti-L-selectin antibody (Mel 14) can influence CTL sensitization and metastatic colony formation. Mel 14 can inhibit CTL activity. Establishment and growth of metastatic colonies of tumor cells expressing these antigens (sLex, sLea) are inhibited when anti-sLea or anti-sLex antibodies are administered to mice in a pancreatic tumor murine model [143]. To facilitate a cellular response, the authors had designed peptide 106, a peptide mimetic of sLex. MHC-binding prediction calculations identified an H2-K^d and I-A^d binding motiv centered on the RYDIYWRYDI sequence of the 106 peptide. $(RYDIYWRYDI)_8$-MAP has been prepared and used to immunize mice. In all cases, QS-21 has been used as adjuvant. Immunization with the MAP mimetic of sugar constituents of neolactoseries antigens induced a MHC-dependent peptide-specific cellular response that triggers IFN-γ production, correlating with IgG2a induction. They observed also an enhancement in CTL activity *in vitro* against sLex-expressing Meth A cells.

Retinoblastoma (RB) protein is a tumor suppressor gene product with a central role in transcriptional control of cell differentiation. Therefore, RB is a common target for many proteins with growth regulatory

activity [173]. A tetrameric MAP $(LFYKKVGGG)_4$-K_2-K-G-OH containing the sequence 649–654 of the RB has been prepared using SPPS methods. Numerous modifications of the synthetic protocol have been examined, including a combination of the Boc and Fmoc approach, use of DBU as Fmoc deprotection agent, Wang-resin, PAM-resin, etc. Best results were obtained on PAM-resin, when the first three Gly were incorporated by Boc chemistry and the remaining AAs were used as Fmoc derivatives. No biological activities have been given.

Apoptosis

The E2F family of transcription factors is a key factor in the regulation of cell proliferation. E2F are heterodimeric proteins consisting of one member of the E2F family and one member of the DP family. The E2F family members interact with specific proteins of the pRB family (pocket proteins). The interaction with pocket proteins results in the repression of E2F-dependent transactivation in the early G1 phase of the cell cycle. In contrast, free and transcriptionally active E2F prevail as cells proceed from G1 into the S phase of the cell cycle. The central role played by E2F and its correlation with cell proliferation and tumorigenesis makes it a promising target to control cellular proliferation. Two different peptides have been isolated from two random phage display libraries that specifically interfere with the function of the transcription factor E2F [142]. These peptides specifically interact with the DNA-binding domain of E2F family members without interfering with DP-1 heterodimerization, and thereby block DNA binding of E2F-DP dimers, both *in vitro* with recombinant proteins and *in vivo*. The relatively low inhibitory activity of the peptides has been increased up to 100-fold when the peptides have been used as tetravalent MAPs. The MAPs have been conjugated to a HTV-Tat-derived membrane-penetrating peptide known to translocate through the plasma membrane and to localize to the nucleus. A Tat fragment encompassing AAs 48–60 and covering the complete basic domain retains full translocation activity. The Tat fragment fused to the *C*-terminal of the MAP is capable of delivering these branched molecules into cells. This is strongly suggested by the ability of these MAPs to reduce the expression of E2F-regulated genes, to inhibit cell proliferation and to induce apoptosis. These MAPs can be useful leads for the development of therapeutic peptidomimetics for the treatment of proliferative diseases.

Lysozyme Epitopes

The effectiveness of synthetic peptide immunogens derived from immunodominant T-cell epitopes as replacements for their intact parent protein in vaccines has been studied [168]. Fluorescein (FL) has been conjugated to hen egg lysozyme (FL-HEL, positive control) and to three synthetic peptide immunogens: (i) murine B10.A (H-2(a)) immunodominant T-cell epitope of HEL [FL-(T-cell epitope)]; (ii) MAP with this epitope $[FL\text{-}(T\text{-epitope})]_n$-MAP; $n = 2–4$ and (iii) negative control with T-cell epitope residues replaced with glycine $[(FL\text{-}Gly_{18})_4$-MAP]. The dose–response has been examined over a 300-fold range in B10.A mice. The FL-(T-cell epitope)'s immune response was dose dependent, with maximum response comparable to that of $[FL\text{-}(T\text{-epitope})]_n$-MAP, or FL-HEL. $[FL\text{-}(T\text{-epitope})]_n$-MAP's immune response was consistently high and nearly dose independent, a trend observed across 1-, 2- and 3-degree responses. $(FL\text{-}Gly_{18})_4$-MAP did not elicit an immune response except at the highest dose. The immunogenicity of the monomeric epitope was 300-fold lower than its parent carrier protein. The MAP constructs increased the immunogen valency and compensated totally for reduced potency. The authors conclude that a specific immunodominant T-cell epitope sequence for HEL is necessary for successful peptide mimicry of HEL.

Canine Erythropoietin (EPO)

The determination of canine erythropoietin (EPO) concentration is crucial for monitoring the effect of human recombinant EPO therapy in dogs with chronic renal failure. The authors [110] developed a simple and sensitive ELISA for canine EPO. This could serve for developing a commercially available assay. The ELISA was based on a mouse MAb and a rabbit PAb using gene electrotransfer to generate the PAb and MAPs to generate the MAb.

Complexation with Ca^{2+}

SLE is an autoimmune disease with autoantibodies directed against ribonucleoproteins as a characteristic feature of the disease. Ro ribonucleoprotein is one such target of these autoantibodies. MAPs constructed from 60-kDa Ro sequence could be used to show intra- and intermolecular protein–protein interaction within the 60-kDa Ro ribonucleoprotein particle. The authors [103] hypothesized that this interaction might be mediated by divalent metal ions. When purified 60-kDa Ro was incubated with various metal ions such as Cu^{2+}, Mg^{2+}, Zn^{2+} and Ca^{2+}, and analyzed by Ouchterlony or surface plasmon resonance for binding to specific 60-kDa Ro-MAPs, only Ca^{2+} ions significantly increased the binding. It was interesting that recombinant 60-kDa Ro formed precipitin lines with Ro-MAPs only in the presence of Ca^{2+} ions. Anti-Ro60 containing SLE sera bound to recombinant Ro60 strongly when incubated in the presence of Ca^{2+} ions, but not in their absence. The obtained data imply that Ca^{2+} induces a more native tertiary structure to recombinant 60-kDa Ro and makes

it more antigenic. Thus, 60-kDa Ro is a calcium-binding protein.

Transport of Na^+ Across 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine Liposomes

Peptide dendrimers with cores from 1,4-diaminobutane, spermidine and spermine containing lysine branches have been prepared by convergent synthesis. First, both amino groups of lysine have been acylated with cholic acid and then the resulting lysine-dicholamide has been used to acylate the amino groups of the core. In this way, dendrimers with 4, 6 and 8 cholic acid residues, respectively, have been prepared [102]. The transport of Na^+ across 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine liposomes as a model system (because this lipid closely resembles those that are found in natural membranes) has been studied. The exponential increase in activity, on going from the tetravalent to the octavalent dendrimer (10^5 increase), further implies that cooperative forces play a major role in pore formation. The study has provided the strongest evidence to date for a barrel–stave structure. The given structures are not true MAPs, but peptide dendrimers.

Interaction with Cationic Guests

A series of MAPs with crown ether core and lysine branches have been synthesized. Their ability to interact with cationic guests has been investigated using NMR and MS techniques. These supramolecular assemblies have been disassembled by the addition of potassium cations thereby achieving controlled release of the template back into the solution and indicating the potential of this system for reversible encapsulation and release of functional species. The use of ditopic ammonium cations possessing long alkyl spacer chains as templates has also been investigated. In this case, the 2 : 1 assembly that forms goes on to achieve higher order levels of organization, hence gelating the solvent (Figure 17) [79].

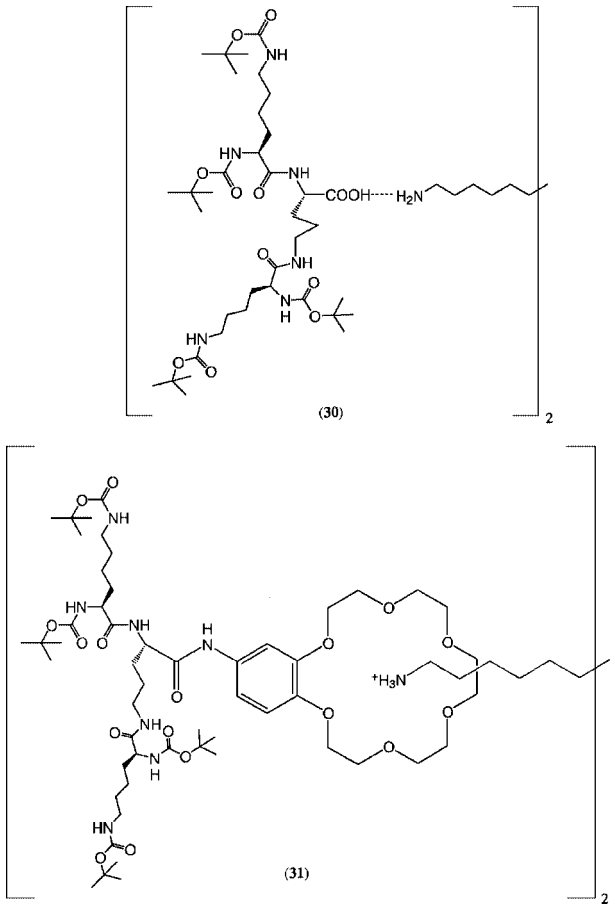

Figure 17 Two-component supramolecular dendritic gels **30** and **31** based on either acid–amine or crown–NH_3^+ interactions between the components [79].

Substances Affecting Platelet Function

Platelet aggregation plays a fundamental role in hemostasis. Lebetins from *Macrovipera lebetina* snake venom constitute a new class of inhibitors of platelet aggregation. There are two groups of peptides: lebetin 1 (L1; 11–13 mer) and lebetin 2 (L2; 37–38 mer). The short lebetins represent the *N*-terminal segments of the longer ones [123]. These peptides inhibit platelet aggregation induced by various agonists (e.g. thrombin, collagen). The lebetins displayed strong *in vitro* antiplatelet activity and were able to prevent collagen-induced thrombocytopenia in rats. Several peptides have been prepared in MAP form. These MAPs have been found to be 1000-fold more active than the corresponding peptides (sL1γ peptide NKPPKKGPPNG) in inhibiting rabbit thrombin-induced platelet aggregation, with IC_{50} values ranging from 2 to 25 pM. The mechanism of antiplatelet action of lebetin remains to be elucidated.

Proline MAPs

Complexation of ciprofloxacin. Giralt *et al.* [90] established efficient synthetic protocols for the preparation of polyproline dendrimers on the basis of a convergent SPPS strategy. Fmoc-Pro_n-L-Amp(Fmoc-Pro_n)-OH ($n = 5$, 14) building blocks have been assembled on two different orthogonally protected cores: spermidine and cyclic Lys-Lys (2,5-DKP) using PyAOP as coupling reagent. This approach demonstrates the suitability of convergent SPPS for the preparation of these compounds and a new method for the synthesis of other peptide dendrimers. The obtained branched polyproline peptides and dendrimers with $n = 14$ have been analyzed by CD. They can adopt both PPII and PPI conformations. Acetyl groups at the periphery

of dendrimers make PPI formation difficult. Fluorescence spectroscopy experiments proved an interaction of dendrimer $(Pro_{14})_2$Amp-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH ←Amp ←$_2(Pro_{14})$ and ciprofloxacin (a synthetic 6-fluoroquinolone antibiotic currently in clinical use for the treatment of infections by Gram-positive and Gram-negative bacteria) in 99.5% propanol with a 1 : 2 stoichiometry and an association constant of 2.0×10^6 M^{-1}. The complex decomposes in water. These results show the possibility of using polyproline dendrimers as new drug delivery systems.

Collagen mimetics. Other proline-rich dendrimers (not MAPs, because the core and branches are nonpeptidic) with nine $(Gly\text{-}Pro\text{-}Nleu)_6$ or $(Gly\text{-}Nleu\text{-}Pro)_6$ motifs have been studied as collagen mimetics. The triple helicity of all structures was determined by thermal denaturation monitored by optical rotation and CD measurements. Mimetics prepared from the $(Gly\text{-}Nleu\text{-}Pro)_6$ sequence form more thermally stable triple helices in comparison to equivalent structures from the $(Gly\text{-}Pro\text{-}Nleu)_6$ sequence for all of the molecules prepared [101].

Recommended Literature on Dendrimers and MAPs in General:

Dendrimers: [2,16,18,19,57];
MAPs: [9–12,54].

CONCLUSIONS

Since 1988, when Tam published his paper about MAPs [53], this work has been cited more than 850 times. This shows that the idea is fruitful and still brings inspiration. New papers on MAPs suggest mainly medical and immunological applications. As can be seen from Tables 2 and 3, most papers are devoted to vaccine development against *P. falciparum* and HIV. The methodology of MAP synthesis has changed from stepwise SPPS to ligation strategies, which are carried out with free soluble prepurified fragments in solution. Therefore, the purification and characterization of the ligated products is much easier than earlier. All these developments lead to defined, pure products and therefore unambiguous results can be achieved.

Acknowledgements

This work was supported by grant No. 203/03/1362 of the Grant Agency of the Czech Republic and Research Project Z40550506.

Citations which are reviews are indicated by '[A Review]'.

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