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Peptide-Decorated Dendrimers and Their Bioapplications

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conjugation chemistry · dendrimers · drug delivery · multivalency · peptides

Peptide-decorated dendrimers (PDDs) are a class of spherical, regular, branched polymers that are modified by peptides covalently attached to their surface. PDDs have been used as protein mimetics, novel biomaterials, and in a wide range of biomedical applications. Since their design and development in the late eighties, poly-L-lysine has been a preferred core structure for PDDs. However, numerous recent innovations in polymer synthesis and ligation chemistry have re-energized the field and led to the emergence of well-defined peptide dendrimers with more diverse core structures and functions. This Minireview highlights the development of PDDs driven by significantly improved ligation chemistry incorporating structurally well-defined peptides and the emerging use of PDDs in imaging and drug development.

1. Introduction

Dendrimers are defined as polymeric macromolecules with a layered architecture that provides a platform for surface chemical modification.[1] A typical dendritic structure consists of three distinctive components: the inner core, the branching units covalently attached to the inner core, and the functional surface groups that predominantly determine the physical and chemical properties of the dendrimer. As a result of the highly branched architecture and a diverse selection of surface functionalities, dendrimer-based systems have applications in a variety of fields, including catalysis,[2] biomedicine,[3] and materials science.[4] Various building blocks have been used to build dendritic structures, such as amidoamine for polyamidoamine (PAMAM) dendrimers,[5] propyleneimine for polypropyleneimine (PPI) dendrimers,[6] L-lysine for poly-L-lysine (PLL) dendrimers,[7] and polyglycerol for polyglycerol (PG) dendrimers (Figure 1).[8] A wide range of ligand types, including truncated antibodies[9] and carbohydrate analogues,[10] have been conjugated to dendrimeric scaffolds as surface functional groups. In this Minireview we focus on peptide-decorated dendrimers (PDDs) that are composed of a polymer core, such as those shown in Figure 1, and contain multiple copies of functional peptides covalently attached to the surface layer. The advantage of PDDs derives from having fine control over their size and the number of copies of attached peptides—a direct result of precise chemical design.[11]

PDDs are most commonly used in the design of biomaterials, drug/gene delivery, and vaccine technology.[12] By integration of the properties of dendrimers and bioactive peptides, PDDs may introduce important synergistic effects, including:[13] 1) a polyvalent structure that increases the biological activity of the attached peptides; 2) amplification of peptide functions by enabling simultaneous interactions with multiple receptors; 3) a protein-like structure that can mimic the action of a variety of biological molecules; 4) biocompatibility and biodegradability; and 5) resistance to proteolysis and delayed renal clearance which may address the short half-lives typically observed in peptide therapeutics.

In this Minireview, we describe a selection of chemical synthesis routes that capitalize on diverse conjugation chemistry (Section 2) and discuss some emerging bioapplications (Section 3) for PDDs.

2. Synthesis of PDDs

2.1. Divergent Conjugation Chemistry

Synthetic routes to PDDs have been extensively explored in the past three decades. Their synthesis may be undertaken either in a stepwise manner (divergent) or by ligation of prepurified peptide segments (convergent) to the dendrimer

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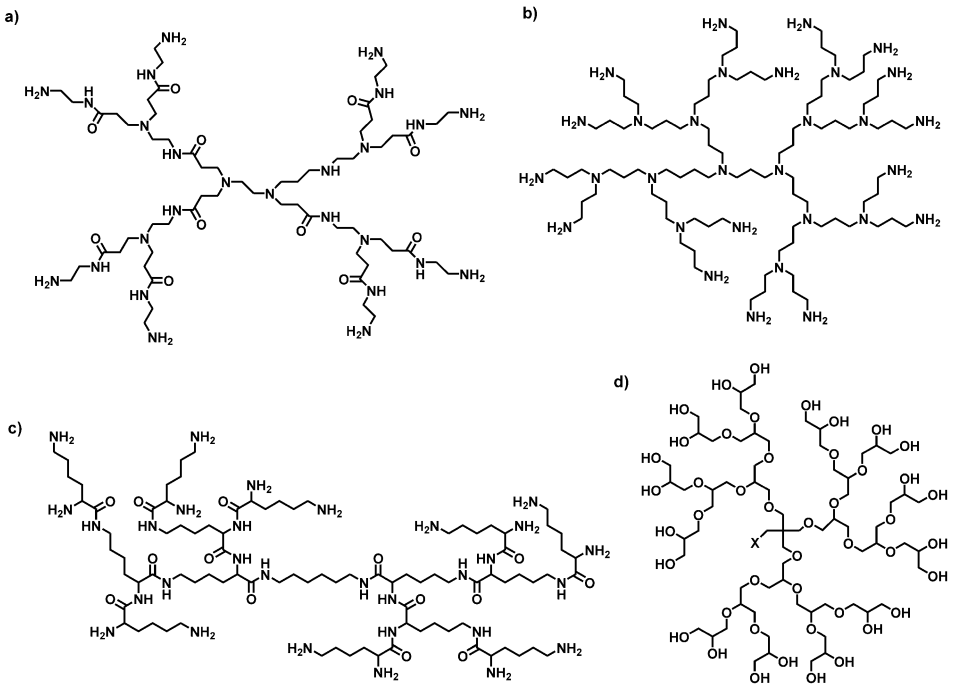

Figure 1. a) A PAMAM dendrimer with amidoamine as the building block, b) a PPI dendrimer with propyleneimine as the building block, c) a PLL dendrimer with L-lysine amino acid as the building block, and d) a PG dendrimer with polyglycerol as the building block.

core. Peptide dendrimers obtained from the divergent route commonly use polyamino acids (such as poly-L-lysine) as branching units and the surface-attached peptide sequences are assembled onto the dendrimer core in a stepwise manner either by solid-phase peptide synthesis (SPPS) or by solution approaches. The pioneering work by Tam in 1988 described the multiple antigenic peptide (MAP) scaffold based on SPPS and PLL as a novel approach to prepare peptide immunogens.[14] In the original chemistry employed, the MAP scaffold was composed of an inner core constructed by layers of lysine residues with a surface layer of peptides chains attached to the inner core. Solid-phase chemistry utilizing *N*,*N*′-dicyclohexylcarbodiimide (DCC) coupling governed the efficiency of this approach (Figure 2). This "divergent" approach, although easier to manipulate, is offset by difficulties in achieving quantitative coupling for dendrimers with high peptide loading.[15] On the other hand, convergent routes can bypass minor coupling difficulties by allowing purification of the core dendron as well as the peptide segments prior to conjugation. In this way, the convergent route results in the preparation of more homogeneous final products, thus making it an attractive alternative in the design of PDDs.

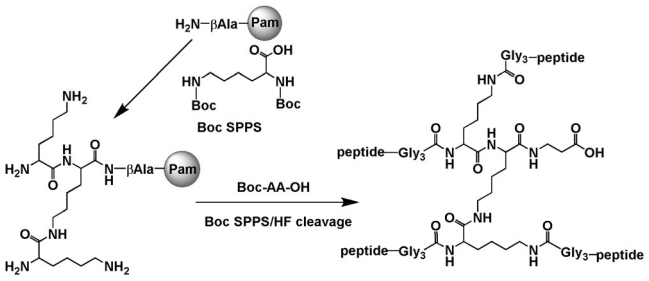

Figure 2. Steps in the synthesis of a tetramer MAP by the stepwise Boc solid-phase method. Gly_3 is the spacer segment, introduced to increase the accessibility of the peptide to the core. Boc = *tert*-butoxycarbonyl. Pam = oxymethylphenylacetamidomethyl.[14]

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2.2. Convergent Conjugation Chemistry

Conjugation between peptides and dendrimers has been widely studied[12b,16] as it promises to yield fully decorated scaffolds, potentially maximizing their biological activity while ensuring homogeneity and reproducibility.[17] However, although complex dendrimers have been achieved, for example in the development of sugar arrays,[18] decoration with peptides has proven to be more challenging, especially for well-structured peptides, such as cyclic and disulfide-rich peptides.[19] To decorate dendrimer end-groups with biologically relevant peptides, it is of crucial importance to employ efficient conjugation chemistry to ensure complete attachment of the ligands to the dendrimer to yield a well-defined product. To achieve this, a key requirement for ligation of unprotected peptides is regio- and chemoselectivity, so that only the desired sites can react with each other in the presence of other peptide side-chain functional groups. Several different ligation methods have been applied to the synthesis of PDDs containing linear or cyclic peptides. For linear peptides, the ligation methods include the formation of hydrazones,[20] oximes,[21] thiazolidines,[22] thioethers,[23] triazoles,[16] and amide bonds by native chemical ligation (NCL).[24] Cyclic peptides dendrimers are less common but are a class of emerging interest.

2.2.1. Dendrimers Carrying Linear Peptides

There are many chemical ligation approaches available for the multivalent presentation of peptides. In 1995, Tam and Shao developed a chemical ligation strategy that makes use of the reaction between a weak base and an aldehyde group to produce tetravalent dendrimers carrying the linear peptide epitope V20.[20] Weak bases, including thiols, hydrazides, and aminooxy groups, are attached to the N terminus of the selected peptide and are ligated with an aldehyde tetrameric PLL dendron under acidic conditions; selectivity is achieved as the basic side-chains are protonated under the conjugation conditions (Figure 3). A similar strategy was applied to the design of a 4-mer multiple antigen peptide by Patarroyo et al., who demonstrated that thiazolidine formation proceeded faster and with greater yield than for oxime or hydrazone formation.[21] Although the ligated MAP dendrimers contain multiple deletions, pure tetramer could be achieved through reverse-phase HPLC (RP-HPLC) purification. To achieve higher generations of the dendrimers, Lambert and co-workers optimized the oxime-forming reaction using keto dendrimers and aminooxy peptides as reaction partners.[22] Thus, PAMAM keto dendrimers with 8 branches were ligated with a 20-residue linear peptide to form dendrimeric mixtures carrying 4, 5, 6, 7, and 8 copies of the linear peptides, as confirmed by gel-permeation HPLC and gel electrophoresis experiments. Further attempts to enhance the degree of peptide loading were unsuccessful.

Thioether ligation involves a typically irreversible reaction and takes advantage of the conjugation of two purified chemoselective components: a haloacetylated (typically chloro) dendron core and a synthetic peptide containing a cysteine residue at either the carboxy or amino terminus

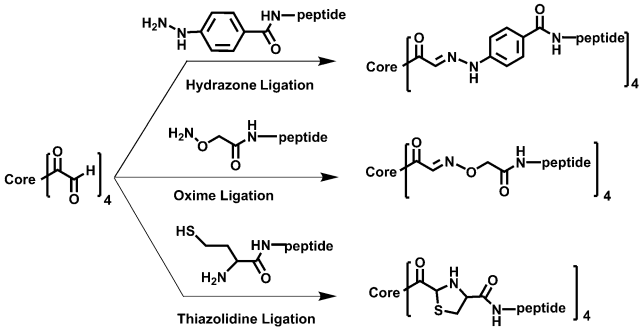

Figure 3. Synthesis of tetramer peptide dendrimers with formation of hydrazone, oxime, and thiazolidine linkages. The core is a tetravalent PLL dendrimer and the attached linear peptide is V20 containing 20 amino acid residues.[20]

(Figure 4a). For example, a 20-residue linear peptide R-HL4 containing a cysteine residue at its C terminus was introduced onto chloroacetylated (ClAc) PAMAM dendrimers by thioether ligation yielding homogeneous 4-mer and 8-mer peptide dendrimers. Attempts to generate homogeneous 16-mer and

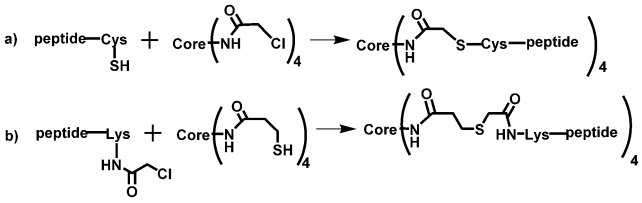

Figure 4. Thioether-based routes to build tetrameric peptides. In approach (a), peptides containing Cys at the C terminus reacted with a ClAc-functionalized PAMAM dendron core. In contrast, using route (b), the reaction was performed reversely with the attachment of ClAc-peptides to the thiol-functionalized PAMAM dendron.[25]

32-mer peptide dendrimers failed.[23] Nevertheless, this ligation method stands out for its simplicity, good yields, and the metabolic stability of its products. One concern when employing thioether ligation chemistry is that dimerization of the peptide by cystine formation may compete with thioether formation. To address this concern, a revised version of this strategy was proposed (Figure 4b) whereby the chloroacetyl-derivatized peptide epitope is conjugated to a thiol-functionalized PLL dendron core in the presence of an excess of the reductant tris(2-carboxyethyl)phosphine (TCEP). The group of de La Torre reported a successful ligation with this approach in an aqueous solvent to give homogeneous thioether di- and tetravalent peptide dendrimers.[25]

Native chemical ligation (NCL) was first developed by Kent, Dawson, and co-workers as a novel chemoselective method to ligate two unprotected peptide fragments to form a native peptide bond.[26] This reaction occurs between a peptide with a C-terminal thioester and a peptide with an N-terminal cysteine residue under aqueous conditions at neutral pH and takes advantage of the much greater solubility and purity of unprotected peptides. Many applications of NCL involve the chemical synthesis of small proteins[27] and

the immobilization of peptide and proteins onto nanoparticles.[28] NCL was further extended by Meijer and co-workers to a dendrimer platform[24] where homogeneous 4-mer and 8-mer PAMAM dendrimers carrying the linear peptide LYRAG were obtained. Attempts to generate the homogeneous 16-mer dendrimer failed and MALDI-MS analysis showed a mixture of 12 to 16 copies on the dendrimer surface. This group also designed asymmetric dendrimers with an arithmetic branching scheme where cysteine dendritic wedges containing a single chemically addressable group (called the focal point) and two, three, four, or five branches were synthesized (Figure 5). Such molecules can incorporate various imaging modalities (such as biotin or chromophores) at the focal point in addition to 2–5 copies of linear thioester peptides at the dendrimer surface (Figure 5).[29]

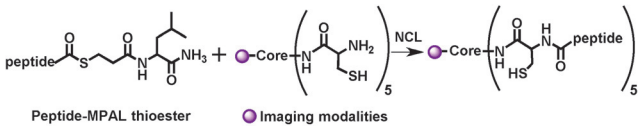

Figure 5. The synthesis of an asymmetric polyamide dendron carrying a linear peptide and imaging modalities by native chemical ligation.[29] The imaging modalities can be biotin or chromophores.

Although NCL and thioether ligation serve as powerful tools for peptide ligation, one major limitation of these approaches is the utilization of the thiol functional group. Given the importance of cysteine residues in peptides, the introduction of the free thiol moiety may cause unpredictable conformational changes of the peptides, for example, disulfide bond shuffling or undesirable by-products from non-selective thiol ligation. Accordingly, alternative synthetic platforms that are more chemoselective have emerged.

Copper-catalyzed azide–alkyne cycloaddition (CuAAc)[30] is the most widely applied type of click chemistry[31] and has been rapidly embraced for applications in bioconjugation.[12b,13a] The CuAAc reaction possesses many properties that distinguish it from alternative coupling strategies, such as high yields, tolerance of reaction conditions (for example, pH and solvent), formation of a single product, a rapid rate, and most importantly its high chemoselectivity.[32] The azide and alkyne groups that are the reaction partners in the CuAAc reaction are inert to other functional groups under typical conjugation conditions and are highly suitable for the immobilization of molecules such as peptides that contain multiple functional groups. Therefore the CuAAc reaction is an ideal chemical tool for the quantitative chemoselective ligation of unprotected peptides and dendrons.

Liskamp et al.[16] reported an efficient microwave-assisted synthesis of multivalent dendrimeric peptides using the CuAAc reaction. A series of azido peptides of different lengths were reacted with increasing generations of alkyne dendrimers in the presence of $CuSO_4$/Na ascorbate/Cu wire in a variety of solvent systems using microwave irradiation. Conjugation between the four-residue linear peptide and the G_1 dendrimer with four branches was readily achieved, although the coupling failed for higher generation dendrimers. More recently, Toth et al.[12b] designed a vaccine delivery system consisting of a polyacrylate core decorated with the linear B-cell epitope J14. The starting materials were a polyacrylate alkyne dendrimer with eight branches and N-terminal azido-peptide J14, and the reaction was carried out in the presence of copper wire at 50 °C. Elemental analysis suggested the conjugation of five peptide epitopes to the dendritic core. In contrast, Galdiero et al. reported the functionalization of an 18-mer azido-polyamide-based dendrimer with a 20-residue alkyne modified linear peptide gH625 in the presence of $CuSO_4$ and sodium ascorbate.[33] The authors claimed that complete functionalization of the dendrimer with peptides was obtained, as the azide stretch disappeared in IR spectroscopic analysis of the peptide dendrimer. Supporting evidence such as NMR and MS characterization was lacking in this study.

Strain-promoted azide–alkyne cycloaddition (SPAAc) has become an attractive alternative of CuAAc, especially in the synthesis of materials targeting biomedical applications.[34] A potential drawback of the classical CuAAc reaction has been the cytotoxicity of the Cu^I catalyst.[35] In the SPAAc reaction, cyclooctynes with intramolecular strain were used that can readily react with an azide with high yields in the absence of a metal catalyst. SPAAc has been used to image biomolecules in living systems[36] and in functionalization of PAMAM dendrimers with ethylene glycol chains.[37] Anseth and co-workers further applied this reaction in the ligation of an azido–PEG dendrimeric scaffold (PEG = polyethylene glycol) with a 16-mer peptide containing two difluorinated cyclooctyne groups at the end chains; the fully loaded scaffold subsequently formed a hydrogel in 1 h (Figure 6).[38]

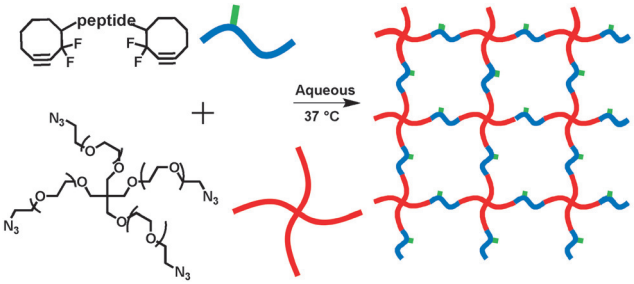

Figure 6. The SPAAc reaction between tetra-azide poly(ethylene glycol) and a linear peptide modified with two difluorinated cyclooctyne groups at the end chains, forming a 3D-network ideal hydrogel. (Adapted from Ref. [38] and reprinted with permission from Macmillan Publishers Ltd, [*Nat. Mater.*], copyright (2009)).

2.2.2. Dendrimers Carrying Cyclic Peptides

In contrast to linear peptides, cyclic peptides possess well-defined ligand structures and have been reported to cause significant steric hindrance to dendrimer conjugation.[19a] Two main categories of cyclic peptides have been investigated, specifically N- to C-cyclic peptides and disulfide-bond-containing peptides.

Cyclic arginine–glycine–aspartic acid sequences (cRGDs) are implicated in cellular attachment through integrin recep-

tors $\alpha_v\beta_3$, and have applications in drug delivery,[39] gene delivery,[40] imaging,[41] and cancer therapy.[42] cRGD peptides have been used to decorate synthetic scaffolds with multiple branches to enhance cellular attachment and increase tumor uptake.[43] Amide bond conjugation chemistry is the most widely used in the conjugation of cyclic RGD and various dendrimer platforms.[44] Kessler and co-workers[44c] have described an Fmoc SPPS-based chemical approach (Fmoc = 9-fluorenylmethoxycarbonyl) for the synthesis of multimeric cyclic RGD peptides possessing two and four copies of cRGD peptides on a PLL dendron. A radiolabeled precursor was then attached to the dendron focal point by oxime ligation in solution for targeting purposes.

Fréchet and co-workers employed a pentaerythritol dendrimer with higher peptide loading.[41] They functionalized eight dendritic branches with a heterogeneous bifunctional PEG chain to provide sufficient flexibility for multiple ligand binding. The PEG chains were coupled to glutaric anhydride and then converted into active imidazolides. Coupling of these imidazolides to cyclic RGD peptides through a pendant lysine amino group led to an average attachment of five cRGD units per dendrimer. One reason for the difficulties in achieving homogeneous multimers primarily lay with an inefficient acid–amide conjugation. To overcome the incomplete conjugation reaction, Wängler et al. investigated three different "click" reactions, specifically CuAAc, oxime, and thiol–maleimide ligation between PAMAM dendrimers and cRGD peptides, and determined the limits of cRGD multimerization.[45] Homogenous cRGD multimers containing one, two, four, eight, and sixteen moieties could be obtained on PAMAM dendrimers by means of a Michael addition of thiols to maleimides.

Peptides with well-defined tertiary structures commonly have disulfide bonds in their frameworks.[46] In 2003, Andreu and co-workers incorporated a cyclic epitope with one disulfide bond onto the lipidated PLL core by peptide bond formation in solution, to yield a mixture containing a maximum of three copies of the cyclic disulfide epitope.[47] To fully load the tetramer dendron branches with a cyclic disulfide peptide, van Leeuwen et al.[48] built a glutamic acid based dendron carrying an additional β-alanine as a spacer segment to increase accessibility for the chemical ligation and to aid multiple receptor binding. The Ac-TZ14011 cyclic peptide containing one disulfide bond was fully loaded onto the scaffold by peptide bond formation that generated a homogeneous tetrameric cyclic peptide dendrimer.

Click chemistry is an attractive approach for the conjugation of cysteine-containing cyclic peptides with a functionalized dendron. In 2009, Liskamp and co-workers designed and synthesized a series of peptide dendrimers carrying the Tyr^3-octreotide peptide through a CuAAc reaction between the peptidyl azides and dendrimeric alkynes using CuOAc as the catalyst.[49] This strategy led to monomeric, dimeric, and tetrameric peptide dendrimers, as confirmed by LC–ESIMS.

Similar to linear peptide conjugation, $CuSO_4$ and sodium ascorbate may also be utilized in the CuAAc conjugation of azido disulfide bond containing peptides and alkyne dendrimers. This method was demonstrated by our group to prepare modified α-conotoxin ImI (α-ImI; an α7-nicotinic receptor blocker) containing two disulfide bonds with PEG spacers on the N-terminal and alkyne-modified PLL dendrimers in the presence of $CuSO_4$, sodium ascorbate (low concentration), and the ligand TBTA (Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine; Figure 7).[50] LC–ESIMS analysis confirmed the successful formation of homogeneous di- and tetravalent dendrimers incorporating the α-ImI peptide.

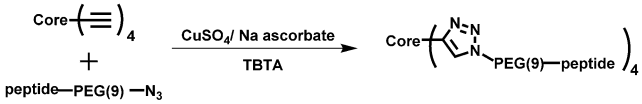

Figure 7. Synthesis of tetramer dendrimers carrying an oxidatively folded peptide through the CuAAc reaction.[50] The core is a tetravalent PLL dendrimer and the attached cyclic peptide is α-ImI containing two disulfide bonds.

With significant optimization, thiol–maleimide chemistry can also be utilized in the design of cysteine-containing cyclic peptide dendrimers (Figure 8). In the study by Hackeng et al., a peptide containing a disulfide bridge and an N-terminally added thiaproline-GlyGly linker was prepared.[19b] Subsequently, the thiaproline was converted into cysteine for the purpose of coupling to the maleimide-functionalized dendron core (Figure 8a). The major concern of this strategy is the isomerization of the peptide through disulfide shuffling in the presence of a thiol; nevertheless, this can be overcome either by replacing the flexible linker by a more rigid spacer or simultaneously performing thiol unlocking and thiol–maleimide coupling (Figure 8b).

Oxime ligation was also used in the design of tetrameric dendrimers carrying the 9-amino acid peptide, LyP-1, containing one disulfide bond.[51] Each N terminus of the

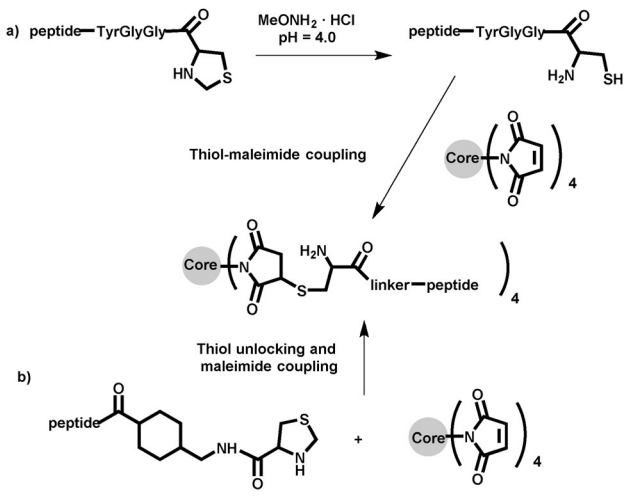

Figure 8. Synthesis of multiple antigenic peptides incorporating oxidatively folded peptide epitopes through thiol–maleimide chemistry.[19b] The core is a tetravalent PLL dendrimer and the attached cyclic peptide contains one disulfide bond.

tetrameric dendrimers was modified with an aminooxy group to conjugate the oxidatively folded peptide LyP-1 bearing a ketone at the C terminus. Using this method, the fully loaded tetrameric $(LyP\text{-}1)_4$–dendrimer was obtained, and was subsequently characterized by MALDI-TOF MS.

Although these convergent systems have demonstrated considerable success, their limitations in the synthesis of higher generation PDDs cannot be ignored. For example, to date no octameric dendrimers with disulfide bonds have been reported. Even for the small cyclic RGD peptides, the largest homogeneous PDDs synthesized are 16-mer dendrimers.[45] It appears that efficient chemistry needs to be developed to achieve larger dendrimers with fully loaded peptides.

3. Bioapplications of PDDs

3.1. Imaging

Important imaging modalities applied to PDDs include radionuclide-based imaging and fluorescence-based optical imaging where many peptide ligands have been functionalized with radioactive and/or fluorescent labels to attain receptor imaging.[52] However, care must be taken as labeling peptides often causes a drop in peptide binding and function and may also alter their biodistribution.[53] Peptide multimerization that increases the amount of peptide on the dendrimer surface with respect to the introduced label could reduce the influence of the label on activity and enhance binding affinity. Several research groups have applied the multimerization concept to prepare peptide dendrimers consisting of multiple peptides and hybrid labels to improve specificity. For example, the cyclic RGDfK dimer and tetramer, NH_2-Glu[c(RGDfK)]$_2$ and NH_2-GluGlu$_2$[c-(RGDfK)]$_4$, labelled with ^{18}F through the N-succinimidyl-4-^{18}F-fluorobenzoate ([^{18}F]SFB) prosthetic group on the focal point, have been used to develop integrin $\alpha_V\beta_3$ targeted radiotracers for tumor imaging by single-photon emission computed tomography (SPECT) and positron emission tomography (PET)[44a,54] where it was found that the tetramer ($IC_{50} = 15$ nM) had slightly better integrin $\alpha_V\beta_3$ binding affinity than its dimeric analogue ($IC_{50} = 32$ nM). The fluorescent label 5(6)-carboxyfluorescein (FAM) was successfully anchored to the C-terminal amino group in a PLL dendrimer carrying four copies of the cyclic 9-residue peptide LyP-1.[51] The in vitro fluorescence images depict an increased accumulation and penetration of the $(LyP\text{-}1)_4$–dendrimer–FAM as compared to the monomer LyP-1–FAM.

Dual-function PDDs with fluorescent probes and chelates for radionuclide attachment were created as robust macromolecular multimodal imaging agents. Kuil et al.[48] prepared mono-, di-, and tetrameric dendrimers containing the Ac-TZ14011 peptide (that targets the CXCR4 chemokine receptor) and functionalized the dendrimers with a multimodal label, consisting of a Cy5.5-like fluorophore and a diethylenetriaminepentaacetate (DTPA) chelate (Figure 9). The labeled dimer and tetramer had higher affinity compared to the labeled monomer and exhibited a lower affinity than the unlabeled monomer peptide. All three dendrimeric peptides were successfully used to image the CXCR4-receptor-expressing tumor.[48]

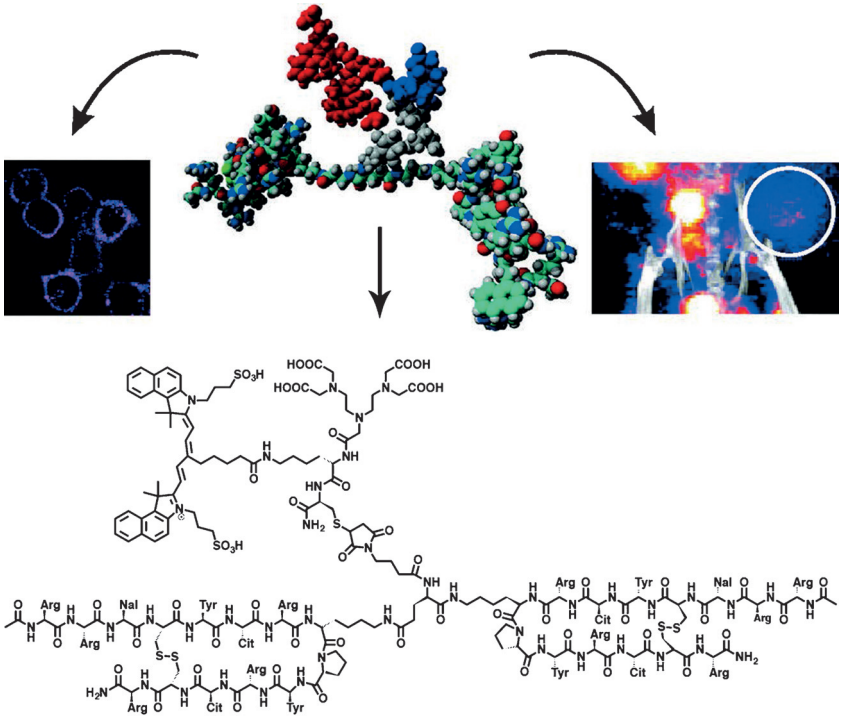

Figure 9. Dimer peptide dendrimers carrying Ac-TZ14011 peptides and a multimodal label for confocal microscopy and SPECT/CT imaging. (Adapted from Ref. [48], copyright (2011), with permission from the American Chemical Society.)

3.2. Drug Delivery

Dendrimers have been extensively used as drug/gene carriers over the past few decades.[55] PDDs can deliver such cargo through targeted receptor binding.[56,57] For example, PEGylated PAMAM dendrimers with cyclic RGD peptide ligands and carrying the anticancer drug doxorubicin (DOX) conjugated by the acid-sensitive *cis*-aconityl linkage show active targeting through specific recognition between RGD and integrin $\alpha_v\beta_3$, passive targeting by the PEG–PAMAM polymeric carrier, and controlled release of free DOX in the presence of weakly acidic lysosomes.[39] Shah et al. constructed a multifunctional drug delivery system (DDS) employing a modified PPI dendrimer as the carrier for the anticancer drug paclitaxel (as a cell-death inducer) plus a synthetic analogue of luteinizing hormone-releasing hormone (LHRH) peptide (to target the tumor).[58] Treatment with this DDS platform led to the efficient induction of cell death, effective tumor shrinkage, and prevention of adverse side effects on healthy organs.

Multifunctional nanomaterials for theranostics have attracted considerable attention recently.[59] For PDDs, Taratula and co-workers reported a multifunctional theranostic platform, in which PEGylated PPI dendrimers were conjugated with LHRH peptides through a thiol–maleimide reaction, and a hydrophobic drug phthalocyanine (with strong absorption in the far-red and NIR) was physically captured in the PPI dendrimer framework for imaging and photodynamic therapy.[60] The imaging experiments revealed that an efficient internalization into cancer cells and tumor accumulation could be achieved with this nanocarrier when intravenously administered into mice. RGD-peptide-modified dendrimers carrying both fluorescein isothiocyanate (FI) through a thiourea linkage and the drug DOX through physical encapsulation were designed by Shi et al. (Figure 10a).[61] Fluorescent imaging confirmed the specific internalization of the PDD carrier in cancer cells with overexpressed integrin $\alpha_v\beta_3$ and the captured DOX drug was then released in a sustained manner.

PDDs can also act as stimuli-responsive materials in drug delivery. Enzymatically cleavable and temperature-sensitive peptides have been attached to dendritic scaffolds for controllable drug release. Haag and co-workers built a scaffold containing hyperbranched polyglycerol as a carrier, dipeptide FK or tetrapeptide AFKK as an enzyme-degradable linker, and doxorubicin or methotrexate as the loaded drug.[62] The conjugate could release the drug in the presence of cathepsin B overexpressed in tumor cells and displayed antiproliferative activity against two human tumor cell lines. Thermosensitive elastin-like peptides with a VPGVG repeat have been conjugated to PAMAM dendrimers to design an elastin-mimetic dendrimer for drug delivery purposes,[63] although detailed studies in a biosystem are still lacking.

Amphiphilic peptide–dendrimer conjugates that can self-assemble into nanostructures are a novel class of drug delivery system. Gu et al. designed mPEGylated peptide dendrimer–GFLG–DOX conjugates through a two-step efficient click reaction that were able to self-assemble into nanoparticles with a particle size of around 80–100 nm

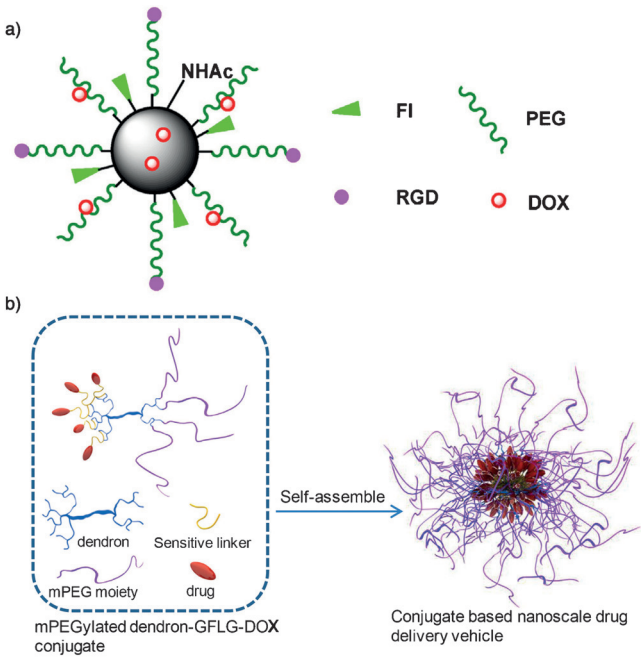

Figure 10. Schematic representation of a) the G5 PAMAM.NHAc–FI–PEG–RGD/DOX complexes and b) amphiphilic mPEGylate dendron–GFLG–DOX conjugates self-assembled into nanoparticles. (Reprinted from Refs. [61] and [64b], copyright (2015) and (2014), with permission from Elsevier.)

(Figure 10b).[64] The GFLG peptide, which can be cleaved by cathepsin B, was explored as an enzyme-responsive linker to connect the anticancer drug DOX. Those nanoparticles showed higher tumor accumulation as a result of the enhanced permeability and retention (EPR) effect, low toxicity and side effects because of the controllable drug release, and gave higher antitumor activity in vivo in mice with ovarian and breast cancer models when compared to free DOX at an equal dose.

Specific delivery can also be realized in gene studies. For instance, a triblock dendritic nanocarrier, PAMAM–PEG–cRGD, was developed and studied as a siRNA vector targeting the human ether-à-go-go-related gene (hERG) in human anaplastic thyroid carcinoma cells.[40] The PAMAM–PEG–cRGD conjugates exhibited negligible cytotoxicity as a result of the conjugation of the PEG chain and enhanced cellular uptake through RGD–integrin recognition and downregulated the expression of hERG to 26.3% of the control value. Liu et al.[65] decorated PAMAM dendrimers with dual-function peptides containing RGDK (for specific tumor homing) and the E_{16} peptide (for promoting cell penetration through interaction with neuropilin-1 receptors). The peptide-modified dendrimers were mixed with Hsp 27 siRNA to form complexes through electrostatic interactions. The complexes led to two-fold higher gene silencing compared to the nontargeted delivery in vivo and potent anticancer activity by inhibiting the tumor growth in prostate cancer models both in vitro and in vivo.[65] A glycine-terminated polyglycerol dendron was modified with a hydrophobic C-18 alkyl chain at the focal point of the dendron, and the constructed amphiphilic dendron was then assembled to

aggregate as micelles for nonviral gene delivery.[66] The gene vector is nontoxic even at high N/P ratios (nitrogen to phosphate ratio N/P = 100) and can efficiently deliver siRNA to achieve potent gene silencing in HeLa cells.

3.3. Macromolecular Drugs of Functionalized Dendrimers

Dendrimers are not only used as drug carriers but may also be fabricated to generate vaccines, antiviral agents, antitumor therapeutics, and pharmacological probes of ion channels. Traditionally, antigenic peptides require conjugation to a protein or peptide carrier to induce a strong immune response in order to overcome the difficulties of weak antigenicity and avoid complications from immunogenic carrier proteins. Tam developed multiple antigenic peptides (MAPs)[14] which are composed of PLL dendrimers with the antigenic peptide being covalently attached. The synthesized MAPs with multiple copies of the antigen largely strengthened their immunogenicity and led to promising vaccines for various diseases, including aphtae epizooticae,[67] AIDS,[68] and malaria.[69] Recently, Gervay-Hague et al. prepared multivalent peptide constructs carrying two different peptide components that can stimulate simultaneously B-cell and T_h-cell immune responses.[12a] The hetero-multimeric peptide constructs displayed enhanced binding, avidity, and specificity toward an established HIV-neutralizing human antibody Mab b12, making it a potential HIV-1 vaccine candidate. Interestingly, PDDs with an amphiphilic structure can self-assemble to produce nanoparticles that consist of a peripheral antigenic epitope layer conjugated to a dendrimeric core.[12b] To develop a novel vaccine against group A streptococci (GAS) with decreased autoimmunity, a dendritic structure consisting of a polyacrylate core and a peripheral generation of the minimal B-cell epitope J14 was self-assembled to generate 20 nm nanoparticles in water (Figure 11), thus allowing maximum possible exposure to the immune system and producing a strong immune response to the GAS M protein.

Peptide dendrimers may also be used as antiviral drugs. For example, Sato et al. synthesized carbosilane dendrimers carrying three, four, and six copies of hemagglutinin (HA) binding peptide (Ala-Arg-Leu-Pro-Arg), respectively.[70] The 6-mer PDD showed the strongest inhibitory activities against two human influenza viruses, A/PR/8/34 (H1N1) and A/Aichi/2/68 (H3N2), both with an IC_{50} value of 0.6 μM.[70] A membrane-interacting alkyne-modified peptide derived from the herpes simplex virus (HSV) type 1 glycoprotein H was attached to a polyamide-based azido dendrimer by Weck and co-workers.[71] This peptide dendrimer was likely to interact with the viral envelope glycoproteins in both HSV-1 and HSV-2, preventing the virus from coming into contact with cellular membranes. The IC_{50} values for HSV-1 and HSV-2 were 100 and 300 nM, respectively, with no evident cell toxicity at these concentrations.

Recently, Gu and co-workers made tryptophan-rich peptide dendrimers as a novel dendritic peptide drug for tumor therapy.[72] The PDDs were assembled from a polyhedral oligomeric silsesquioxane (POSS) core, a lysine backbone, and a terminal tryptophan group using solution-phase peptide synthesis. Owing to the abundant tryptophan residues that contain indole rings, the therapeutic dendrimers displayed significant supramolecular interactions with DNA. Interestingly, the dendrimers showed superior cytotoxicity towards various tumor cell lines and also inhibited the proliferation of tumor cells in vivo and accelerated cell apoptosis at tumor sites. Inhibition of splicing factors of pre-mRNA, such as FBP21 protein, has become of interest in the field of antitumor drugs.[73] Freund et al. constructed by acid–amide conjugation a multivalent dendritic polyglycerol scaffold carrying on average seven peptides with the sequence WPPPPRVPR.[74] These PDDs displayed a circa 10-fold enhanced affinity toward the WW domains of FBP21 protein with respect to the monovalent peptide, providing an approach to the inhibition of the proline-rich sequence recognition of FBP21.

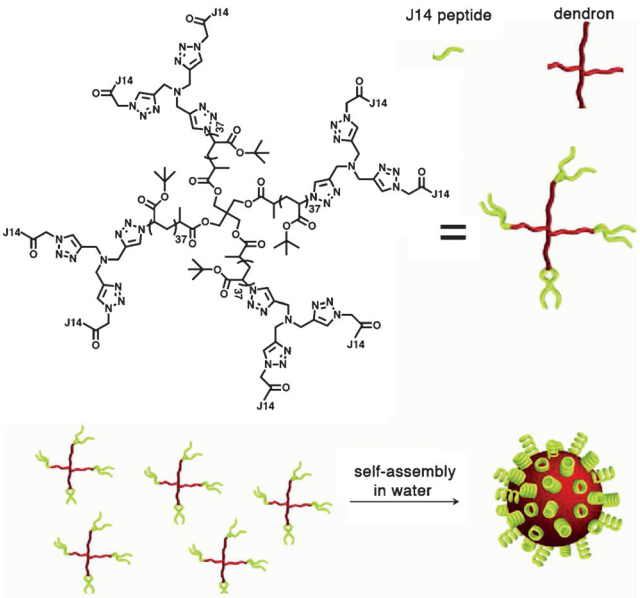

Figure 11. Synthesis of polyacrylate dendrimer nanoparticles by self-assembly. (Adapted from Ref. [12b], copyright (2010), with permission from John Wiley and Sons.)

We have designed peptide dendrimers exhibiting enhanced pharmacological activity toward the homomeric nicotinic acetylcholine receptor (nAChRs; Figure 12).[50] Homomeric di- and tetrameric lysine dendrons carrying the α7-nicotinic receptor blocker α-ImI were synthesized using click chemistry. A spacer PEG-9 was introduced between the α-ImI peptides and the dendron branches to realize concomitant binding to multiple binding sites. The dimeric ImI construct had the highest potency at hα7 nAChR (IC_{50} = 4 nM), which is enhanced by about 100-fold compared to native α-ImI (IC_{50} = 440 nM); no significant activity enhancement was detected at the heteromeric hα3β2 and hα9α10 nAChRs. Hence, dendrimer design can serve as a promising tool to improve the potency and selectivity of bioactive peptides.

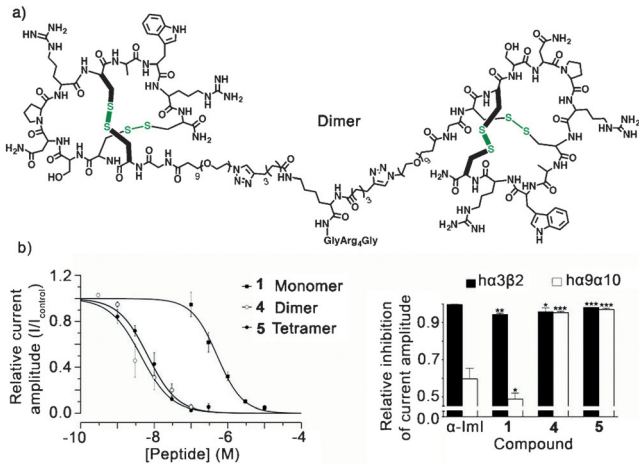

Figure 12. a) The dimeric peptide dendrimer carrying conotoxin α-ImI and b) inhibition of hα7, hα9α10, and hα3β2 nAChR subtypes by α-ImI and ImI dendrimers. (Adapted from Ref. [50], copyright (2015), with permission from the American Chemical Society.)

3.4. Protein Mimetics

Dendrimers are similar in size to biomolecules, such as small proteins and enzymes, and may be designed to mimic the action of a variety of such molecules. PDDs, such as dendrimeric collagen, have proven to be useful protein mimetics.[75] In 2008, Tong et al. appended two types of trifunctional peptides onto the surface of PAMAM dendrimers to form collagen mimetic dendrimers.[76] The collagen-mimetic peptides are composed of: 1) a repeating GPO (Gly-Pro-Hyp) sequence to mimic the triple helical structure of collagen; 2) a cell-binding sequence Gly-Phe-Hyp-Gly-Glu-Arg (GFOGER); and 3) either Ala-Pro-Gln-Gln-Glu-Ala (APQQEA) as an amine acceptor probe or Glu-Asp-Gly-Phe-Phe-Lys-Ile (EDGFFKI) which acts as an amine donor substrate for tissue transglutaminase (tTGase; Figure 13). The two types of peptide dendrimers were then mixed in a 1:1 ratio and enzymatically cross-linked producing a supramolecular structure that exhibited a stable collagen-like triple helical conformation and improved cellular recognition. Interestingly, Kono et al. designed a thermosensitive collagen-mimic drug carrier by combining collagen peptides (Pro-Pro-Gly)$_5$ with a fourth generation PAMAM dendron by amide bond formation.[77] The collagen dendrimers induced the formation of a collagen-like triple helix that was thermally reversible, in contrast to natural collagen. By adjusting dendrimer generations and peptide length, these dendrimers can be used as a potential cellular matrix for controllable drug release.

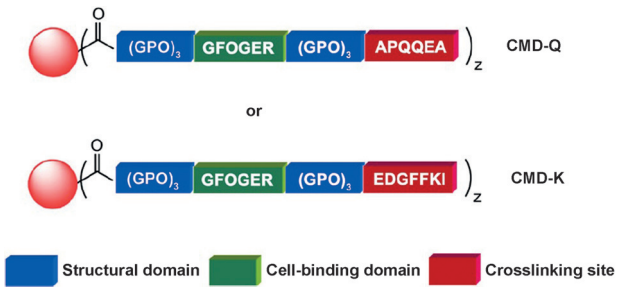

Figure 13. PAMAM dendrimers containing two types of collagen-mimetic peptides. (Reprinted from Ref. [76], copyright (2008), with permission from Elsevier.)

4. Summary and Outlook

Polymer scientists have made significant progress in the synthesis and use of new materials for biomedical applications.[78] PDDs that exhibit properties of both the peptides and the dendrons are especially attractive. In this Minireview, we described examples of novel synthetic routes that generate PDDs and selected bioapplications of PDDs.

Improvements in the synthesis of PDDs have relied heavily upon chemoselective peptide ligation chemistry to conjugate either linear or cyclic peptides. Nevertheless, full decoration of higher generation dendrimers with well-structured peptides remains a challenge. Besides the optimization of ligation chemistries and the development of more efficient conjugation chemistry, the role of spacer segments between conjugated peptide and dendrons should be further investigated with a view to increasing the loading capacity of PDDs in higher generation dendrimers. Moreover, the length and flexibility of such spacers needs to be varied accordingly when oligomeric receptors are targeted to achieve multiple binding (multivalency).[79]

Reflecting the development of the early MAPs system, PDDs were initially used as vaccines and antiviral agents. More recently, they have been extensively used for specific receptor targeting for imaging and drug-delivery applications. The integrin $\alpha_V\beta_3$ receptor is one of the better known targets where multiple presenting cRGD peptides have enhanced their affinity towards integrin $\alpha_V\beta_3$. G-protein-coupled receptors (GPCRs) have also attracted considerable attention in multivalent design, though in most cases little significant affinity enhancement has been found when compared to the monomer peptide ligand. Further optimization studies should investigate whether the spacer length and peptide orientation can lead to successful multivalency. Very recently, our group found that dendrimeric peptides had significantly enhanced affinity and functional activity at an ion channel, the α7 nicotinic acetylcholine receptor (nAChRs).[50] This finding might be further extended to a wide variety of ion channels leading to more potent and selective ligands.

PDDs are now beginning to be explored more methodically, particularly in the field of biomedicine. However, heterogeneity remains a significant issue and full characterization of PDDs is sadly lacking in many cases. In sum, chemically optimized synthetic routes, better structural data, improved quality control analyses, and a better grasp of biophysical properties are necessary if PDDs are to become more promising candidates in many clinical applications.

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