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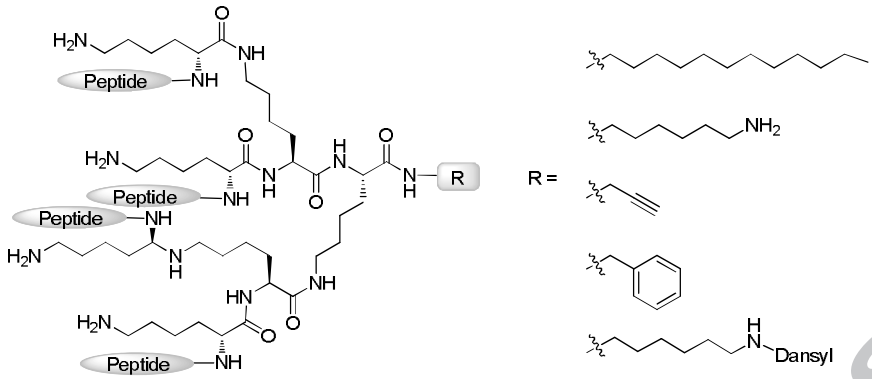


Solid-phase synthesis of polyfunctional polylysine dendrons using aldehyde linkers

Daniel K. Svenssen[a], Sahar Mirsharghi,[a] Ulrik Boas[a, b *]

[a] Innate Immunology Group, National Veterinary Institute, The Technical University of Denmark. Bülowsvej 27, DK-1870 Frederiksberg, Denmark

[b] Center for Nanomedicine and Theranostics, The Technical University of Denmark, Building 345E, Ørsteds Plads, DK-2800 Kongens Lyngby, Denmark

Email: uboa@vet.dtu.dk

Abstract

A straightforward method for the solid-phase synthesis of C-terminally modified polylysine dendrons has been developed by applying bisalkoxybenzaldehyde and trisalkoxybenzaldehyde linkers. The method has been used for the synthesis of polylysine dendrons with a variety of C-terminal 'tail groups' such as alkyl, propargyl and dansyl to give dendrons in high crude purity. Furthermore, the method was successful for the synthesis of dendrons with multiple N-terminal pentapeptide groups together with C-terminal alkyl and propargyl tail groups. Finally, the method was shown to be well-suited for automated synthesis.

Dendrimers and dendrons belong to a class of molecularly defined/monodisperse hyperbranched molecules that present a high number of functional groups at their surface. The high number of functionalitites on the surfaces of dendrons and dendrimers enables these structures to act as high affinity ligands for various biological receptors. Therefore, these structures have great potential in

the development of new biomaterials.[1] Dendrimers and dendrons are categorized both by the monomers they comprise and by the number of branching points following a chain from the core, or in lysine dendrons, from the C-terminal to the periphery (N-terminal). The number of branching points in the chain is denoted by the generation number (Gn) of the dendron.[2] Hence the dendrimer/dendron structure grows larger for each generation in a defined manner.

Dendrimers and dendrons based on the amino acid L-lysine were initially developed by Denkewalter[3] and their application in biological research was pioneered by Tam and coworkers.[4] In this regard, there have been several reports on the synthesis of poly-L-lysine (PLL) dendrons by solid-phase synthesis (SPS), both by Tam's group[5,6] and subsequently by other research groups.[7-10] The reported SPS of PLL dendrons applies a Rink amide or Rink acid linker to give dendrons with a primary amide or carboxylic acid as the C-terminal functional group. However, being C-terminal protective groups, these linkers give only limited possibility to modify the C-terminus of the dendron with an additional functionality during solid-phase synthesis.

The ability to tailor specific polyvalent structures with several functionalities may be valuable in the preparation of, e.g., biologically active compounds. As we are interested in preparing multifunctional dendron ligands for a large variety of receptors, we set out to develop a straightforward strategy for the solid-phase synthesis of a large variety of polyfunctional dendron scaffolds. A peptide linker allowing both C-terminal and N-terminal modification would pave the way for the facile introduction of several functional groups during the synthetic sequence.

Linkers based on bis- and trisalkoxybenzaldehydes such as the backbone amide linker (BAL, with an *ortho* or *para* substituted spacer) and a 4-(4-formyl-3-methoxyphenoxy)butanoic acid (FMPB) linker (Figure 1) were initially developed for the solid-phase synthesis of C-terminally modified peptides. Subsequently, they have found wide use in the synthesis of modified and cyclic peptides as well as small non-oligomeric molecules.[11-14]

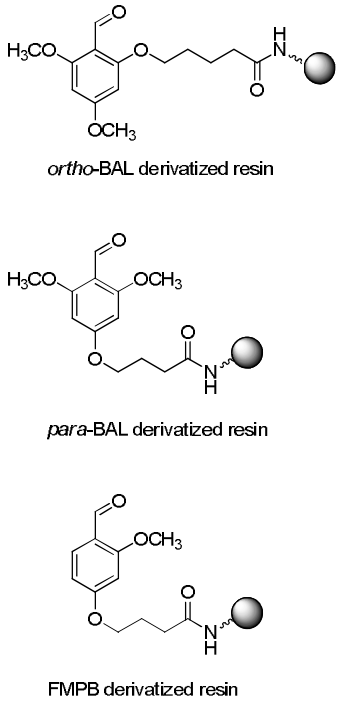
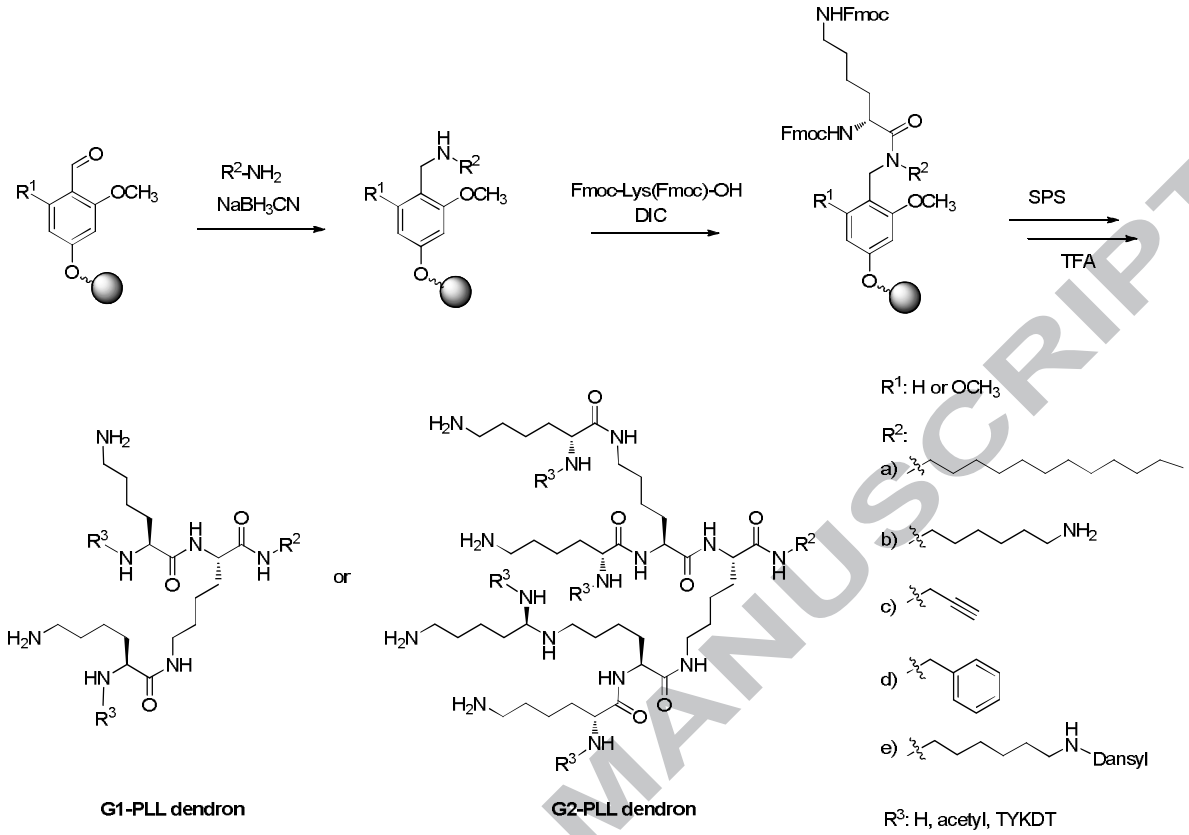


Figure 1. Bis- and trisalkoxybenzaldehyde derivatized aminomethylated polystyrene resins.

In the solid-phase synthesis of dendrons the 'backbone amide linkage approach' enables the introduction of a large variety of C-terminal 'tail' functionalities early in the synthetic sequence, without the need to introduce additional orthogonal protecting groups (Scheme 1). The initial step in the synthesis is the formation of a secondary amine by reductive amination between the linker aldehyde group and a C-terminal functionality which contains an amino group. By subsequent acylation of the secondary amine with lysine, a dendron can be synthesized on the resin by divergent synthesis. Due to stabilization of the alkoxybenzyl carbonium ion, the dendron substrate with the secondary amide anchor point can be released in a 'traceless' manner from the resin under mild acidic conditions (Scheme 1).



Scheme 1. Synthetic sequence for the formation of poly-L-lysine dendrons with a variety of C-terminal groups.

The bisalkoxybenzaldehyde (FMPB) and trisalkoxybenzaldehyde (*ortho-/para*-BAL) linkers[15] were linked to aminomethylated polystyrene resin [1% divinylbenzene (DVB) cross-linked] or high loading rigid macroporous polystyrene (10% DVB cross-linked) by an amide linkage using (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) and diisopropyl amine (DIPEA).[15,16] The introduction of the linker onto the resin was monitored by infrared spectroscopy (attenuated total reflectance). Here an aldehyde band appeared at approximately 1715 cm^{-1} (rigid macroporous resin) or at approximately 1670 cm^{-1} (1% DVB cross-linked resin) together with an amide band (approx. 1650 cm^{-1} for the rigid macroporous resin and 1600 cm^{-1} for the 1% DVB cross-linked resin). Hence, a red-shift was generally observed with the 1% DVB cross-linked

resin compared to the rigid macroporous resin.[17,18] The presence of aldehyde groups on the resin was furthermore visualized colorimetrically by either the purpald test[19] (where the presence of resin aldehyde groups gave purple beads), or by reaction with 2,4-dinitrophenylhydrazine (DNPH) in sulfuric acid (orange-red to deeply red beads with resin aldehyde groups).[20] The loading of linker groups on the resin was reduced to avoid 'crowding' on the resin during synthesis of the more bulky dendrons.[21] The dendron C-terminal functionality was introduced onto the aldehyde linker resin via reductive amination using sodium cyanoborohydride in a dipolar aprotic solvent (DMF or NMP) under mild acidic catalysis (5% acetic acid). In this step, amines with a large variety of functional groups could be introduced. The reductive amination reactions were generally complete within 2-3 hours according to IR and purpald or DNPH tests.

On-resin IR analysis of the rigid macroporous resin after reductive amination of the aldehyde generally showed strong reduction of the aldehyde band (approx. 1715 cm^{-1}) in accordance with the negative DNPH test. In contrast, with the 1% DVB cross-linked resin, only a slight reduction of the aldehyde band (approx. 1670 cm^{-1}) was observed relative to the amide band, although purpald and DNPH tests were negative. However, upon reductive amination with long chain alkyl amines, both resins showed a significantly increased C-H stretching band (approx. 2900 cm^{-1}) relative to the aldehyde band, which was indicative of a successful reductive amination.[17,19,20] With long chain alkyl amines, e.g., dodecylamine, a precipitate formed in NMP, so in these cases, a less polar solvent mixture [THF/NMP (9:1)] was applied to better dissolve the amine during the reaction.

The subsequent acylation of the secondary amine on resin with Fmoc-Lys(Fmoc)-OH was generally performed using *N,N′*-diisopropylcarbodiimide (DIC) twice over two hours (which worked better compared to a 4 hours reaction time). In this procedure, the insoluble *N,N′*-diisopropylurea byproduct precipitated within 10 minutes. This urea byproduct could be removed by washing the resin with MeOH and NMP. By employing the coupling agent fluoro-*N,N,N′,N′*-

tetramethylformamidinium hexafluorophosphate (TFFH) for the acylation of the secondary amine and reaction times two hours (twice), this precipitation could be avoided and similar coupling yields were obtained. Phosphonium or uronium peptide coupling agents such as PyBOP or *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU), did not give complete acylation of the secondary amine under these reaction conditions. For this step the content (loading) of the amines was determined by measuring the UV absorption of the released dibenzofulvene-piperidine adduct (λ=290 nm) from the Fmoc protected amines on the resin. [22] Generally, in the synthesis of dendron substrates, higher loadings (loading ~ 0.3 mmol/g) were obtained with the *ortho*-BAL linker compared to the *para*-BAL and FMPB linkers (loading ≤ 0.1 mmol/g).

The subsequent dendron synthesis was carried out by Fmoc-based SPS. In some cases, Fmoc-Lys(Boc)-OH was applied in the coupling step to release free ε-amines in the dendron product upon acidolytic release of the dendron from the resin. In the divergent build-up of the dendron, only two equivalents of the activated amino acids per amine were applied to afford complete acylation of the terminal amines. This is a lower excess than the 4-5 equivalents generally needed to complete acylations in the synthesis of peptides on solid phase. The acylations were complete within one hour in the formation of G1-dendrons (indicated by a negative ninhydrin test). However, upon stepwise divergent formation of G1- and G2-dendrons carrying N-terminal peptide groups (TYDKT, which is a partial T cell epitope[23]), the coupling times in the synthesis of the peptide sequence usually increased to 2-3 hours before obtaining a negative ninhydrin test. In the synthesis of dendrons with N-terminal TYDKT peptides, delayed coloring during the ninhydrin test was occasionally observed. This led to false negative results after Fmoc deprotection of the amino acids with bulky side-chain groups (e.g., aspartic acid with a *tert*-butyl ester side-chain group, or threonine). However,

inconclusive ninhydrin results could be overcome by prolonged heating during the analysis (5 minutes at 80 ^{o}C), which then gave reliable colorimetric results.

In order to use the method as a tool for C-terminal fluorescence labeling of dendrons we first reductively aminated the aldehyde linker with 1,6-diaminohexane followed by reaction of the primary amine with fluorescein isothiocyanate. However, the introduction of fluorescein at the C-terminus led to complex product mixtures. This may be due to the presence of two phenolic groups and a carboxylic acid on the fluorescein structure, which are prone to reaction during the subsequent synthesis of the dendron. However, two routes to introduce an alternative dansyl fluorophore gave a satisfactory outcome, albeit with somewhat lower crude purities of the products. The dansyl group could either be introduced via reductive amination with a mono-dansyl modified hexane diamine,[24] followed by acylation and Fmoc-based SPS of the dendron. Alternatively, a similar result could be obtained by reductive amination with 1,6-diaminohexane followed by selective dansylation of the primary amine. To increase the selectivity towards the reaction with a primary amine in the presence of a secondary amine, we found it necessary to deactivate the dansyl chloride by reaction with hydroxybenzotriazole (HOBt) to form an intermediate HOBt-sulfonic acid ester.[25]

Amino groups at the dendron surface could be released as free amines or acetylated amines. Also, a triglycine peptide was successfully introduced by segment coupling, opening up possible segment coupling by other peptide sequences comprising a C-terminal glycine residue. The G1- and G2 dendronized triglycine derivatives were synthesized in 37-44% isolated yields possessing either dodecyl or 6-aminohexyl tail groups (Table 1). To further investigate the scope of the present method, the automated synthesis of dendron structures with N-terminal peptide groups was carried out on a peptide synthesizer. Here, we synthesized two G1-dendrons with a C-terminal dodecyl and propargyl tail and two N-terminal peptide groups. G1- and G2-dendrons with a propargyl tail group (**8** and **18**) and with two and four N-terminal TYKDT peptide groups, respectively, were

successfully synthesized by automated synthesis (Table 1). Here, product **18** was of high crude purity according to HPLC-MS, whereas crude product **8** showed a minor impurity with an M + 96 mass. The M + 96 mass could result from either the TFA ester or trifluoroacetamide adduct of **8** formed during the acidolytic cleavage from the resin. The propargyl tail group was chosen, since it allows the introduction of a large variety of functionalities by click chemistry. The G1-dendron with a dodecyl tail group **7** was synthesized both manually and by automated synthesis, the crude product **7** prepared by automated synthesis showed some minor impurities according to HPLC-MS with an M + 96 (TFA ester or TFA amide) together with a minor HPLC peak with mass M – 128. The M – 128 mass was found to be a deletion sequence missing one lysine residue.[17] As deletion byproducts were not observed in the automated synthesis of dendrons with a propargyl group at the C-terminus, this could indicate that the hydrophobic dodecyl chain influences the acylation steps due to steric effects, occasionally giving deletion sequences. Hence, in these cases, the automated synthesis protocol may be further optimized to resolve this problem. However, pure product **7** was obtained by manual solid-phase synthesis. To further determine the structure of the dendronized TYDKT peptides, derivatives **7** and **18** were analyzed by amino acid analysis (AAA) and found to be in accordance with the expected amino acid compositions, $Lys_5Thr_4Asp_2Tyr_2$ and $Lys_{11}Thr_8Asp_4Tyr_4$, respectively.

Table 1

Synthesized dendrons and their isolated yields[24]

	Compound	Yield (%)
1	G1-PLL($(NH_2)_4$)-N-dodecyl	47
2	G1-PLL($(NH_2)_4$)-N-(6-aminohexyl)	72
3	G1-PLL($(NH_2)_2$ $(AcNH)_2$)-N-dodecyl	69
4	G1-PLL($(NH_2)_2$ $(AcNH)_2$)-N-(6-aminohexyl)	52
5	G1-PLL($(NH_2)_2$ $(AcNH)_2$)-N-propargyl	56
6	G1-PLL($(NH_2)_2(AcNH)_2$)-N-(6-dansylaminohexyl)	67
7	G1-PLL($(NH_2)_2(TYKDT)_2$)-N-dodecyl	42
8	G1-PLL($(NH_2)_2(TYKDT)_2$)-N-propargyl	44
9	G1-PLL$(GGG)_4$-N-dodecyl	44
10	G1-PLL$(GGG)_4$-N-(6-aminohexyl)	44
11	G2-PLL($(NH_2)_8$)-N-dodecyl	31
12	G2-PLL($(NH_2)_8$)-N-(6-aminohexyl)	42
13	G2-PLL-($(NH_2)_4(AcNH)_4$)-N-dodecyl	55
14	G2-PLL($(NH_2)_4(AcNH)_4$)-N-(6-aminohexyl)	60
15	G2-PLL($(NH_2)_4(AcNH)_4$)-N-benzyl	35
16	G2-PLL($(NH_2)_4(AcNH)_4$)-N-propargyl	68
17	G2-($(NH_2)_4(GGG)_4$)-N-dodecyl	37
18	G2-PLL($(NH_2)_4(TYKDT)_4$)-N-propargyl	32[a]

[a] A somewhat higher loading (0.11 mmol/g) and a lower isolated yield was obtained due to the lack of capping of the free amine groups on the resin.

In summary, a straightforward method for the preparation of polyfunctional lysine dendrons in good yields and crude purities is presented. The method paves the way for the introduction of a large variety of C-terminal functionalities including molecular labels or functionalities which allow further derivatisation in solution, e.g., by click chemistry. Peptide N-terminal end-groups can be introduced by solid-phase segment coupling or by stepwise synthesis. The method can furthermore be implemented in automated SPS of dendrons.

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16. *Procedure for anchoring the aldehyde linker to aminomethylated polystyrene resin*: the aldehyde linker, e.g., FMPB or *ortho-*/*para*-BAL (1 equiv.), PyBOP (1 equiv.) and HOBt (1 equiv.) were suspended in NMP (approximately 7.3 mL/g resin). However, it was later concluded that HOBt could be omitted from the general procedure. DIPEA (2 equiv.) was added and the mixture was swirled for 5 min at r.t. The mixture was transferred

to a filter syringe/glass container with a glass filter containing the aminomethylated polystyrene resin (0.5 or 1 equiv., loading: 2 mmol/g) and shaken for 1 h at r.t., and then washed with NMP (5x10 mL) and CH_2Cl_2 (5x10 mL). The resin was capped with Ac_2O/DIPEA/CH_2Cl_2 10/5/85 (7-10 mL/g resin) for 2 h, or until the ninhydrin test showed negative. The derivatized resin was washed with NMP (5x10 mL), CH_2Cl_2 (5x10 mL) and MeOH (2x5 mL), and then air-dried followed by drying in vacuo. The IR spectrum was recorded showing bands at 1680 cm^{-1} (C=O stretch, amide), around 1700 cm^{-1} (C=O stretch, aldehyde); positive purpald and DNPH tests were observed.

17. Analytical data including spectra and chromatograms can be found in the Supporting Information
18. A strong IR band from residual acetic anhydride was seen at approximately 1790 cm^{-1} after capping of the rigid macroporous resin and the anhydride proved difficult to remove by washing. Treatment with 20% MeOH/5% DIPEA in CH_2Cl_2 overnight removed the band, but sometimes gave rise to a smaller band at approximately 1770 cm^{-1}, presumably due to a methyl acetate (C=O stretch) formed during the MeOH treatment. However, with the 1% DVB cross-linked resin, no band from residual acetic anhydride was observed. We conclude that the reduced swelling ability of the rigid macroporous resin must somehow have an effect on entrapment of acetic anhydride inside the beads.
19. The purpald aldehyde test was performed as described by Cournoyer, J.J.; Kshirsagar, T.; Fantauzzi, P.P; Figliozzi, G.M.; Makdessian T.; Yan, B. *J. Comb. Chem.* **2002**, *4*, 120-124
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22. Determination of the resin loading was carried out as follows: resin (3 x 5 mg) was transferred to falcon tubes and a solution of piperidine in NMP (1:4, 25 mL) was added and the tube shaken for 30 min along with a reference tube containing only the piperidine solution. Each of the four samples were transferred to a cuvette and the absorbance measured by photospectrometry at 290 nm. The absorbance was used to calculate the loading of the resin according to the formula: loading in mmol/g = 4.76 Abs/mass of resin in mg
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25. Data not shown