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RESEARCH ARTICLE

Synthesis and In Vitro Evaluation of Inherent Properties of L-Glutamic Acid Based Dendritic Lipopeptide Oligomers

Namita Hegde[1] · Kapil Juvale[1] · Bala Prabhakar[1]

Abstract

Purpose The present study reports synthesis, characterization and in vitro evaluation of physicochemical and biological properties of dendritic lipopeptide oligomers comprising L-glutamic acid dendrons and myristoyl tails such that termini of the molecules carry carboxylic ester, carboxylic acid or alcohol functions, which account for nonpolar neutral, polar anionic and polar neutral surfaces, respectively.

Methods Reactions adopted in the current work were fairly rapid, moderately simplified and required fewer coupling reagents. As inherent physicochemical and biological properties depend upon structural details, synthesized compounds were tested for the presence of foaming, nanoparticle formation, antibacterial and anticancer potential, if any.

Results The synthesized nonpolar molecule demonstrated potential to form self-assembled polymeric nanoparticles, whereas the polar molecules demonstrated surfactant-like properties. None of the synthesized molecules demonstrated any inherent antibacterial activity against gram-positive as well as gram-negative bacterial strains, but compound with hydroxyl termini showed anticancer activity hint as a result of preliminary screening.

Conclusion The synthesized molecules demonstrate potential for their application as drug delivery materials and hold scope for further investigations.

Keywords Dendron · Myristoyl · Nanoparticle · Peptide · Self-assemble

Introduction

Dendritic molecular assemblies form oligomeric dendrons at lower generations (G) and polymeric dendrimers at higher generations. Dendrons as well as dendrimers are monodispersed large molecules of specific molecular weights, owing to their stepwise synthesis. Such branched polymeric structures are synthesized in a highly controlled manner, i.e. propagating generations of divalent monomers are attached stepwise to a common central core. Synthesis of dendritic molecules is called convergent if it propagates from the periphery towards the central core, and divergent if the propagation is vice versa [1]. Shape of the lower generation oligomeric dendrons appears conical due to its spread growing only towards one side. Dendrons gradually form a spherical dendrimer structure if bonded covalently and a supramolecular self-assembly if bonded non-covalently, as depicted schematically in Fig. 1. These dendritic systems are often utilized as drug delivery carriers.

Drug loading in dendrimers is achieved either through physical encapsulation within its void spaces or through molecular conjugation with functional groups on its outermost surface. In the case of the self-assembled dendrons, drug molecules are often encapsulated within the formed supramolecular structures. In the process of self-assembly, molecular components of a system spontaneously form ordered aggregates (resulting from weak, non-covalent interactions like hydrophobic interactions and hydrogen bonds) under specific conditions of concentration, temperature, pH, etc. [2].

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✉ Kapil Juvale
kjuvale@gmail.com

✉ Bala Prabhakar
bala.prabhakar@nmims.edu

[1] Shobhaben Pratapbhai Patel School of Pharmacy & Technology Management, SVKM's NMIMS, V. L. Mehta Road, Vile Parle (W), Mumbai 400056, India

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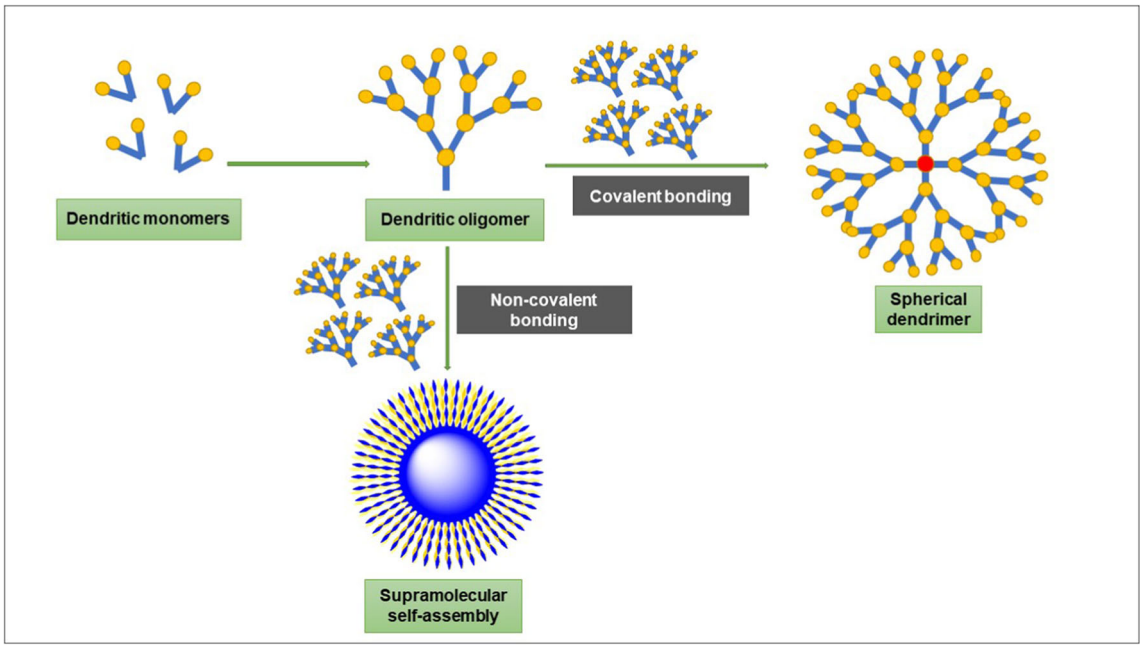


Fig. 1 Schematic representation of covalently and non-covalently bonded oligomeric dendrons

Lipid-linked peptide dendron hybrids are one of the dendritic structures designed to result into supramolecular self-assemblies. Such structures consist of a fatty acid tail linked to a low-generation (G2, G1, or G0) peptide dendron [3]. Lipidic fatty acid chains (e.g. stearic acid, palmitic acid, myristic acid, lauric acid) form hydrophobic portion, whereas the dendritic peptide oligomers constituting di-carboxylic or di-amino (e.g. lysine, arginine, glutamic acid, aspartic acid) amino acids form hydrophilic portion. Self-assembly of such lipopeptide oligomers is driven by interactions between polar and nonpolar portions of respective molecules. Depending upon the type of amino acid present at peripheries, polar ends can carry a surface charge.

Peptide dendrons with positively charged peripheries have been explored extensively for gene delivery and cell penetrating peptide-mediated anticancer applications, as cationic functional groups interact effectively with the negatively charged phosphate groups of cell membranes [4, 5]. In contrast to the aforementioned therapies, such interactions are not required for delivery of small molecule drugs. Cationic peripheries pose a major limitation of inducing haemolysis and cytotoxicity in healthy cells, resulting from their uncontrolled interactions with phosphate groups present in cell membranes of healthy cells. Hence, for the purpose of small molecule drug delivery, dendritic carriers with neutral and/or anionic surfaces should be preferred [6].

Owing to their inherent biocompatibility and biodegradability, the amino acid derived polypeptides as well as the lipid-based nanomaterials are often candidates of choice for drug delivery applications. The hybrid lipopeptide carriers would be suitable for various drug delivery applications, particularly of antimicrobial and anticancer agents. The lipidic component can enhance intracellular localization of drugs by modifying their cell membrane penetration capacity, whereas entrapped drug can be released by metabolising the amide linkages.

Present study reports design and synthesis of low molecular weight lipopeptide oligomers with neutral and/or anionic peripheries. Using multi-step solution phase reactions, dendritic oligomers comprising L-glutamic acid dendrons and myristoyl tails were synthesized. Reactions adopted in the current work were comparatively rapid, moderately simplified and required fewer coupling reagents. Termini of the molecules were designed with nonpolar neutral, polar anionic and polar neutral groups, i.e. analogues having carboxylic ester, carboxylic acid and alcohol functions at dendron surfaces. As inherent physicochemical properties of molecules differ based on structural variations, synthesized compounds were evaluated to understand differences in their foaming and nanoparticle forming capacities. Furthermore, to study the biological characteristics imparted by surface functional groups and charges, the synthesized compounds were tested using in vitro assays to investigate associated inherent antibacterial or anticancer potential, if any.

Experimental

Materials

L-Glutamic acid dibenzyl ester *p*-toluenesulfonate [7, 8] and myristoyl chloride [9] were synthesized in-house as

previously reported in literature. Extra pure grade L-glutamic acid, anhydrous *N*,*N*-dimethylformamide (DMF), trifluoroacetic acid (TFA) and triethylamine (TEA) were purchased from S. D. Fine Chemical Limited (India). Extra pure grade 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDC.HCl) and sodium borohydride ($NaBH_4$) were procured from Sisco Research Laboratories Pvt. Ltd. (SRL) (India). BOC-L-glutamic acid, 4-(dimethylamino)pyridine (4-DMAP), nickel(II)chloride hexahydrate ($NiCl_2.6H_2O$) and thionyl chloride ($SOCl_2$) were obtained from Spectrochem Pvt. Ltd. (India). The analytical grade solvents were procured from Research-Lab Fine Chem Industries (India). Nutrient agar (M001-500 G) was purchased from HiMedia (India). Progress of the reaction was monitored by thin-layer chromatography (TLC) using silica gel 60 F_{254} supported on aluminium plate (Merck). Fourier-transform infrared (FTIR) spectra of the synthesized molecules were recorded using Perkin Elmer Spectrum Two FTIR spectrometer with UATR. Mass spectra of synthesized molecules were recorded using Shimadzu LC-MS-8040 triple quadrupole LC-MS/MS spectrometer having electrospray ionisation (ESI) source. Teledyne ISCO CombiFlash RF was used for sample purification by flash chromatography on RediSep Rf disposable flash columns of 12 g size. Particle size and zeta potential of self-assembled synthesized molecules was determined using Malvern Zetasizer Nano ZS90. Molecular Devices ID3 Multi-mode Plate Reader was used to measure formazan optical density in anticancer assay. The MDA-MB-231 (breast cancer) cell line was procured from National Centre for Cell Science (NCCS) (India).

Methods

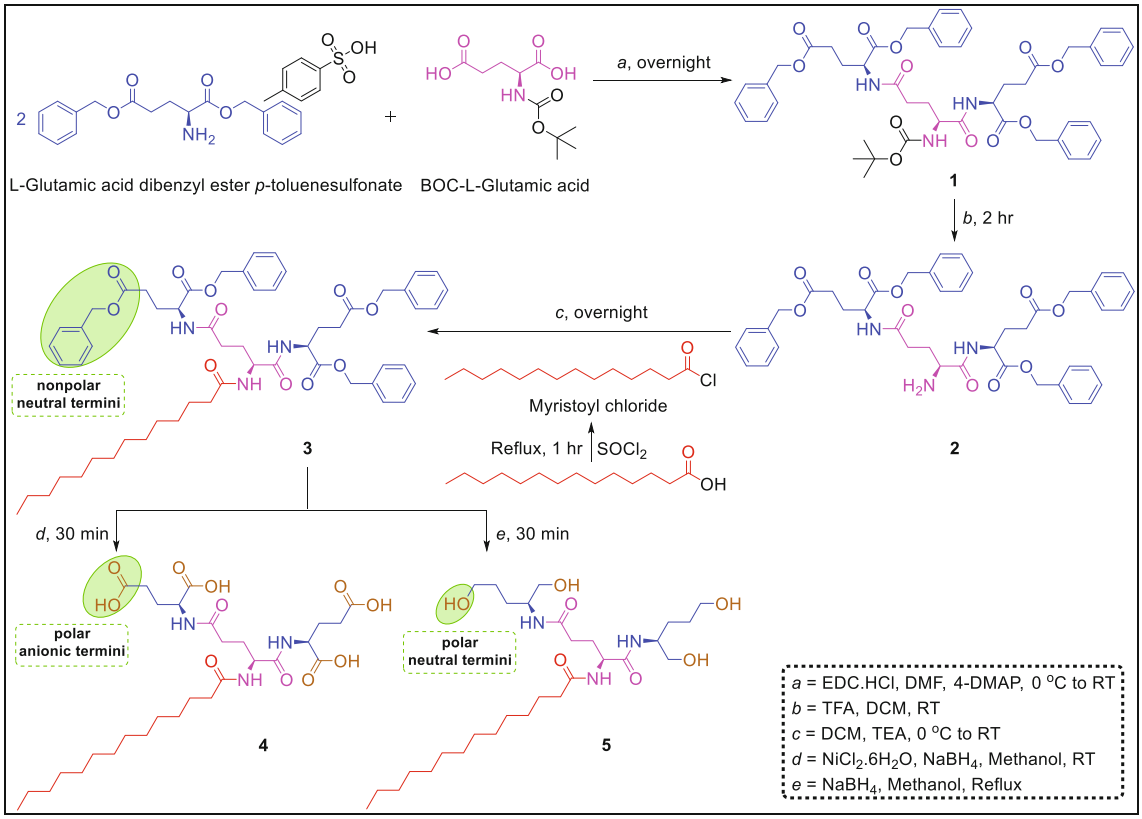
Synthesis of Dendritic Lipopeptide Oligomers

General Procedure for Synthesis of tetrabenzyl 2,2′-(((S)-2-((tert-butoxycarbonyl)amino)pentanedioyl)bis(azanediyl))(2S,2′S)-diglutarate (1) Synthesis of compound **1** was conducted as described in Scheme 1, i.e. BOC-L-glutamic acid was linked to L-glutamic acid dibenzyl ester *p*-toluenesulfonate through an amide coupling reaction. Briefly, 2 g (8 mmol) BOC-L-glutamic acid and 6 g (49 mmol) 4-DMAP was stirred in 25 ml DMF for 15 min at 0 °C under nitrogen atmosphere. To the chilled mixture, 8 g (16 mmol) L-glutamic acid dibenzyl ester *p*-toluenesulfonate and 4 g (20 mmol) EDC.HCl were added and reaction mixture was allowed to gradually reach the room temperature. Stirring was continued at room temperature overnight and progress of reaction was monitored through TLC. On completion, reaction mixture was diluted with 100 ml saturated aqueous citric acid solution and stirred for 1 h. The synthesized compound was then extracted with dichloromethane (DCM). Separated DCM was further washed with saturated aqueous sodium bicarbonate solution and brine, respectively. The resulting organic phase was dried over a sodium sulphate bed and evaporated to dryness under reduced pressure. Yellow thick oil obtained was stored overnight at 0 °C in *n*-pentane for complete precipitation of product **1**. The precipitate obtained was filtered and dried to yield an off-white solid. The obtained crude product was purified using flash chromatography. Mobile phase used was gradient of methanol and DCM such that 100% DCM until 3 min, then gradual increase in polarity to reach of methanol/DCM 5:95 at 8 min and continuation of the same for elution of compound after 9 min.

General Procedure for Synthesis of tetrabenzyl 2,2′-(((S)-2-aminopentanedioyl)bis(azanediyl))(2S,2′S)-diglutarate (2) Deprotection of amine functional group of compound **1** was carried out in the presence of TFA. Briefly, 5 g (5.7 mmol) compound **1** was dissolved in 15 ml DCM at room temperature and 7 ml TFA was added to it in dropwise manner. Resulting mixture was stirred for 2 h and progress of reaction was monitored through TLC. On completion, reaction mixture was quenched with DCM and excess TFA was neutralised by saturated aqueous sodium bicarbonate solution washing. The resulting organic phase was then dried over a sodium sulphate bed, collected and evaporated to dryness under reduced pressure to yield product **2**. Yellow thick oily product obtained was used as amine for next reaction without further purification.

General Procedure for Synthesis of tetrabenzyl 2,2′-(((S)-2-tetradecanamidopentanedioyl)bis(azanediyl))(2S,2′S)-diglutarate (3) Compound **3** was synthesized using acyl chloride of myristic acid, i.e. myristoyl chloride. Briefly, 3.7 g (4.8 mmol) compound **2** was stirred with 0.8 ml (8 mmol) TEA at 0 °C in 10 ml DCM for 30 min. One gram (4 mmol) myristoyl chloride was dissolved in 5 ml DCM and added dropwise to the reaction mixture maintained at 0 °C. Reaction mixture was allowed to reach room temperature gradually, and stirring was continued overnight to yield the product. Progress of reaction was monitored through TLC. On completion of reaction, DCM was evaporated to dryness under reduced pressure. The residue obtained was suspended in water and excess TEA was neutralised with dil. HCl such that pH of solution was maintained between 5 and 7. The obtained crude product was filtered and dried in a desiccator to yield a buffcoloured solid compound **3**. The crude product was purified by column chromatography using DCM/methanol 97:3 mobile phase.

General Procedure for Synthesis of (2S,2′S)-2,2′-(((S)-2-tetradecanamidopentanedioyl)bis(azanediyl))diglutaric acid (4) Benzyl deprotection of compound **3** was performed under mild conditions to yield corresponding carboxylic acid. Briefly, 3.33 g (14 mmol) $NiCl_2.6H_2O$ was dissolved in



Scheme 1 Stepwise synthesis of dendritic lipopeptide oligomers **3**–**5**

15 ml methanol and 1 g (1.02 mmol) compound **3** was suspended in it. Reaction mixture was stirred at 0 °C and simultaneously 1.59 g $NaBH_4$ (42 mmol) was added in small portions. Reaction mixture was allowed to reach room temperature and stirred further for 30 min. Progress of reaction was monitored through TLC. On completion of reaction, resulting mixture was quenched with methanol and filtered through a Celite pad. Filtrate was collected and evaporated to dryness under reduced pressure. The white coloured powder residue obtained was collected as compound **4**.

General Procedure for Synthesis of (S)-N1,N5-bis((S)-1,5-dihydroxypentan-2-yl)-2-tetradecanamidopentanediamide (5) Benzyl deprotection of compound **3** under reflux gave corresponding alcohol. In a two-neck round bottom flask, 1.1 g (1.12 mmol) compound **3** was suspended in 15 ml methanol. Reaction mixture was refluxed and 1.25 g (3.3 mmol) $NaBH_4$ was added simultaneously in small portions. Reflux was continued for further 30 min and progress of reaction was monitored through TLC. On reaction completion, mixture was quenched with methanol. Resulting mixture was evaporated to dryness under reduced pressure and residue was washed with DCM. After extraction, DCM was filtered and filtrate was evaporated to dryness under reduced pressure to obtain compound **5** as a colourless liquid.

Tetrabenzyl 2,2′-(((S)-2-((tert-butoxycarbonyl)amino) pentanedioyl)bis(azanediyl))(2S,2′S)-diglutarate (1) Obtained as a white solid (4.68 g, 68%), R_f (TLC, ethyl acetate/petroleum ether 4:6) = 0.24, IR (KBr, cm^{-1}) 3302.37 (N-H stretching), 1722.42, 1653.59 (C=O stretching), 1600–1450 (aromatic C-C stretching). MS (ESI) m/z calcd for $C_{48}H_{56}N_3O_{12}^+$ $[M+H]^+$ 866.38 found $[M+H]^+$ 866.65, $[M+Na]^+$ 888.60.

Tetrabenzyl 2,2′-(((S)-2-aminopentanedioyl) bis(azanediyl))(2S,2′S)-diglutarate (2) Obtained as a yellow liquid (4.15 g, 95.18%), R_f (TLC, ethyl acetate/petroleum ether 4:6) = 0.023, IR (KBr, cm^{-1}) 3283.65 (N-H stretching), 1733.14, 1672.57 (C=O stretching), 1600–1450 (aromatic C-C stretching). MS (ESI) m/z calcd for $C_{43}H_{48}N_3O_{10}^+$ $[M+H]^+$ 766.33 found $[M+H]^+$ 766.35, $[M+Na]^+$ 788.35.

Tetrabenzyl 2,2′-(((S)-2-tetradecanamidopentanedioyl) bis(azanediyl))(2S,2′S)-diglutarate (3) Obtained as a buff solid (3.65 g, 93.49%), R_f (TLC, methanol/ DCM 1:9) = 0.88, IR (KBr, cm^{-1}) 3286.05 (N-H stretching), 2918.16, 2849.89 (aliphatic C-H stretching), 1736.58, 1635.49 (C=O stretching), 1600–1450 (aromatic C-C stretching). MS (ESI) m/z calcd for $C_{57}H_{74}N_3O_{11}^+$ $[M+H]^+$ 976.53 found $[M+H]^+$ 976.40, $[M+Na]^+$ 998.45.

(2S,2′S)-2,2′-(((S)-2-tetradecanamidopentanedioyl) bis(azanediyl))diglutaric acid (4) Obtained as a white solid (0.53 g, 86.03%), R_f (TLC, methanol/DCM 1:9) = 0.02, IR (KBr, cm^{-1}) 3339.70 (O-H stretching and N-H stretching), 2925.01, 2854.12 (C-H stretching), 1643.98 (C=O stretching). MS (ESI) m/z calcd for $C_{29}H_{50}N_3O_{11}^+$ $[M + H]^+$ 616.34 found $[M + H]^+$ 616.43, $[M + Na]^+$ 638.78; MS (ESI) m/z calcd for $C_{29}H_{48}N_3O_{11}^-$ $[M - H]^-$ 614.33 found 614.58.

(S)-N1,N5-bis((S)-1,5-dihydroxypentan-2-yl)-2-tetradecanamidopentanediamide (5) Obtained as a colourless liquid (0.58 g, 94.15%), R_f (TLC, methanol/DCM 1:9) = 0.02, IR (KBr, cm^{-1}) 3302.19 (O-H stretching and N-H stretching), 2925.79 (C-H stretching), 1659.39 (C=O stretching). MS (ESI) m/z calcd for $C_{29}H_{58}N_3O_7^+$ $[M + H]^+$ 560.42 found $[M + H]^+$ 560.74, $[M + Na]^+$ 582.84, $[M + K]^+$ 598.79.

Determination of Foaming Properties

For determining foaming properties, 1% w/v aqueous solutions of compounds **1**, **3**, **4** and **5** were prepared. Briefly, 10 mg compound was dissolved in 200 μl dimethyl sulfoxide and volume was made to 1 ml with water. Blank solution was prepared similarly. Prepared solutions were shaken manually for 1 min and appearance of foam was observed. Stability of foam was observed for subsequent 15 min.

Determination of Polymeric Nanoparticle Forming Potential

Polymeric nanoparticle formation potential was determined for compounds **3**, **4** and **5** by solvent evaporation method. Briefly, 5 mg compound was dissolved in 200 μl acetone. This mixture was added dropwise through a syringe fitted with needle to 5 ml water maintained at room temperature with stirring. After continuing stirring for 1 h, particle size and zeta potential of resulting mixture were determined.

Antibacterial Assay

Synthesized compounds were screened against bacterial strains of *E. coli* and *S. aureus* using a nutrient agar based assay. Briefly, 1% w/v (10,000 ppm) aqueous test solutions of compounds **1**, **3**, **4** and **5** were prepared as mentioned for determination of foaming properties. Freshly prepared 200 ml nutrient agar media was sterilized in autoclave at 121 °C for 20 min at 15 psig. Liquefied nutrient agar was cooled to 45 °C and approximately 1 ml respective dilute bacterial suspension was added to each flask containing 100 ml nutrient agar. Resulting mixtures were shaken gently and transferred to respectively labelled sterile petri plates. Upon subsequent cooling and solidification of nutrient agar, wells were made and 100 μl of prepared test solutions was added. Ampicillin (500 ppm) solution was taken as a positive control and diluent was taken as negative control.

Anticancer Assay

Anticancer activity related preliminary screening of synthesized compounds was performed by MTT assay in MDA-MB-231 (breast cancer) cell line. MTT assay was carried out as described earlier [10]. Briefly, the cells trypsinised at 90% confluence were seeded (5000 cells/well) into 96-well plate. The plates were then incubated for 24 h at 37 °C with 5% CO_2. Test compounds **3**–**5** were added to the wells in triplicates at 10 μM final concentration, and the plates were incubated for next 72 h. After completion, MTT dye (5 mg/ml) was added to each well and plates were incubated further for 2 h. After discarding the supernatant, 100 μl of DMSO (formazan solubilizing agent) was added to each well. At 570 nm wavelength, optical density of resulting solution was measured. For determining percentage of cell viability, untreated cells were considered as negative control and established anticancer drugs (cisplatin and doxorubicin) were used as standards.

Results and Discussion

Synthesis

Dendritic molecules with neutral and/or anionic surfaces can circumvent the toxicities that otherwise arise from uncontrolled interactions between the cationic termini of dendritic carriers and the phosphate groups present in phospholipid bilayer of healthy cells. Hence, dendritic materials with neutral and/or anionic peripheries should be preferred as carriers for the purpose of drug delivery [6]. In order to develop such material, current work focuses on synthesis of dendritic lipopeptide oligomers comprising L-glutamic acid dendrons and myristoyl chains.

L-Glutamic acid, an amino acid with di-carboxylic acid groups, serves as an excellent monomer unit for growth of dendritic polymers. The carboxylic acid functions can form neutral surfaces when capped with suitable groups and anionic surfaces otherwise. Appoh et al. and Duan et al. attempted synthesis of dendritic L-glutamic acid lipopeptide oligomers and reported molecules with ethyl ester, benzyl ester or carboxylic acid peripheries. Alcohol termini analogues of such molecules have not been found in literature. Moreover, the aforementioned earlier studies reported presence of carboxylferrocene and stearoyl chains at the core, respectively [11, 12]. Compared with the metabolic lipid like stearic acid, a non-metabolite lipid myristic acid has better systemic circulation time [13]. Hence, in the current work, the combination of L-glutamic acid dendrons and myristoyl chains was designed for dendritic lipopeptide oligomers. Corresponding molecules

within the dendritic structures were connected via amide linkages formed by utilizing L-glutamic acid amino groups.

The synthesized dendritic molecules carried benzyl ester (**3**), carboxylic acid (**4**) and alcohol (**5**) functional groups at dendron termini. Amongst these, carboxylic ester and acid termini account for nonpolar neutral and polar charged peripheries, respectively. In order to synthesize dendritic lipopeptide oligomers with polar neutral peripheries, current work included alcohol termini analogues. Furthermore, the reaction conditions adopted in the present work for synthesizing carboxylic ester and acid termini were not only simplified in terms of types of reagents employed, but also rapid. Synthesis was conducted as described in Scheme 1. Synthesis of compound **1** was performed using carbodiimide coupling agent EDC.HCl. It is often a carbodiimide of choice because its nonreactive *N*-acylurea by-product is water soluble, and can be eliminated easily by aqueous washes during work up [14]. Both DCM and DMF are the most widespread solvents used in coupling reactions, the former is neutral aprotic and latter is basic aprotic [15, 16]. We preferred DMF as a solvent since it maintains non-acidic reaction conditions by neutralising HCl separated from coupling agent. Also, instead of 1-hydroxybenzotriazole (HOBt), we used 4-DMAP as a stronger carbodiimide nucleophile activator [17, 18]. Nucleophile activators are coupling additives which form corresponding active esters, preventing conversion of *O*-acylisourea to nonreactive *N*-acylurea [19]. Due to its basic nature, 4-DMAP also helps in activation of acid through proton abstraction. Hence, the presence of DMF and 4-DMAP eliminated need of additional base such as TEA.

Although synthesis of compound **1** as well as compound **3** involved amide coupling, reaction conditions used for former were not suitable for the latter. This was thought to be because of the stearic hindrance offered by benzyl groups of compound **2**. Hence, myristoyl chloride was employed instead of myristic acid for synthesis of compound **3**, as acyl chlorides are more reactive than their corresponding carboxylic acid analogues. On the other hand, HCl produced as a by-product of acyl chloride coupling reactions give rise to acidic reaction conditions. Since such acidic conditions can result into deprotection of BOC-L-glutamic acid [14], acyl chloride mediated coupling was undertaken for synthesis of compound **3** but not for the synthesis of compound **1**.

The reactions catalysed by $NaBH_4$ offer inexpensive, rapid and simple alternative to reduction with H_2, Pd/C catalyst. A reducing agent, nickel boride, was generated in situ for reduction of compound **3** to its corresponding carboxylic acid. Compound **3**/$NiCl_2.6H_2O$/$NaBH_4$ was used in the equivalent ratio of 1:14:42 since reaction involved cleavage of 4 ester bonds [20]. Filtration through Celite pad separates the inorganic salts, making reaction work up simple and rapid. For reduction of compound **3** to its corresponding alcohol, $NaBH_4$ was used as reducing agent. Under harsh reaction conditions of reflux and in the presence of protic solvent like methanol, $NaBH_4$ alone acts as sufficiently strong reducing agent [21]. Compound **5** was extracted in DCM and solid residue containing inorganic salts was separated by filtration.

For synthesis of compound **1**, BOC-L-glutamic acid was used as a di-carboxylic acid and L-glutamic acid dibenzyl ester *p*-toluenesulfonate was used as an amine. Acid, amine, EDC.HCl and 4-DMAP were employed in the equivalent ratio of 1:2:2.5:6.125, respectively. The acid was activated by initial stirring with 4-DMAP. Addition of EDC.HCl at 0 °C slows down the side reaction, which otherwise results into formation of nonreactive *N*-acylurea by-product. Formation of the same is also prevented due to the presence of 4-DMAP, as this nucleophile activator prevents rearrangement of *O*-acylisourea and maintains its reactivity. Addition of deliquescent EDC.HCl under nitrogen atmosphere is required to prevent its inactivation. On reaction completion, 4-DMAP was separated using saturated aqueous citric acid solution. Cold *n*-pentane wash was required for solidification of oily product. The obtained off-white solid product demonstrated the presence of nonpolar impurity in TLC analysis. Hence, the obtained crude product was purified using flash chromatography. The initial mobile phase gradient of 100% DCM resulted into separation of impurity, whereas increase in polarity thereafter resulted into elution of compound after 9 min.

TFA was used to provide mild acidic conditions for deprotection of amine functional group of compound **1**. The crude product obtained is a TFA salt of BOC-deprotected compound **1**. On reaction completion, excess TFA was neutralised to avoid interference of acidic conditions with next coupling reaction. During work up with aqueous sodium bicarbonate, TFA salt breaks and forms an unstable amine. Compound **2** was used immediately in next reaction without further purification to avoid decomposition of free amine. For synthesis of compound **3**, acyl chloride, amine and base were taken in the equivalents of 1:1.2:2. Reduction of compound **3** was simplified and rapid since in contrast to traditionally used H_2, Pd/C catalyst under pressure, $NaBH_4$ was used as reducing agent under atmospheric pressure. Based on the reaction condition, reduction of compound **3** resulted into corresponding carboxylic acid (reflux) or alcohol (room temperature) analogues. Reduction at room temperature resulted into the former, whereas at reflux, it resulted into the latter.

FTIR analysis of synthesized compounds demonstrated in Fig. 2 showed considerable variations in functional group peaks. Significant shift in amine peak from $\nu_{max} \sim 3302$ to $\nu_{max} \sim 3283$ cm^{-1}, respectively, confirmed BOC deprotection of compound **1** and formation of compound **2**. Although both the aforementioned compounds showed presence of aromatic C-H at $\nu_{max} \sim 2900$ cm^{-1} and $\nu_{max} \sim 2850$ cm^{-1}, appearance of sharp C-H stretching bands at 2918.16 cm^{-1} and 2849.89 cm^{-1} confirmed attachment of aliphatic myristoyl group to compound **2** and formation of compound **3**. Due to

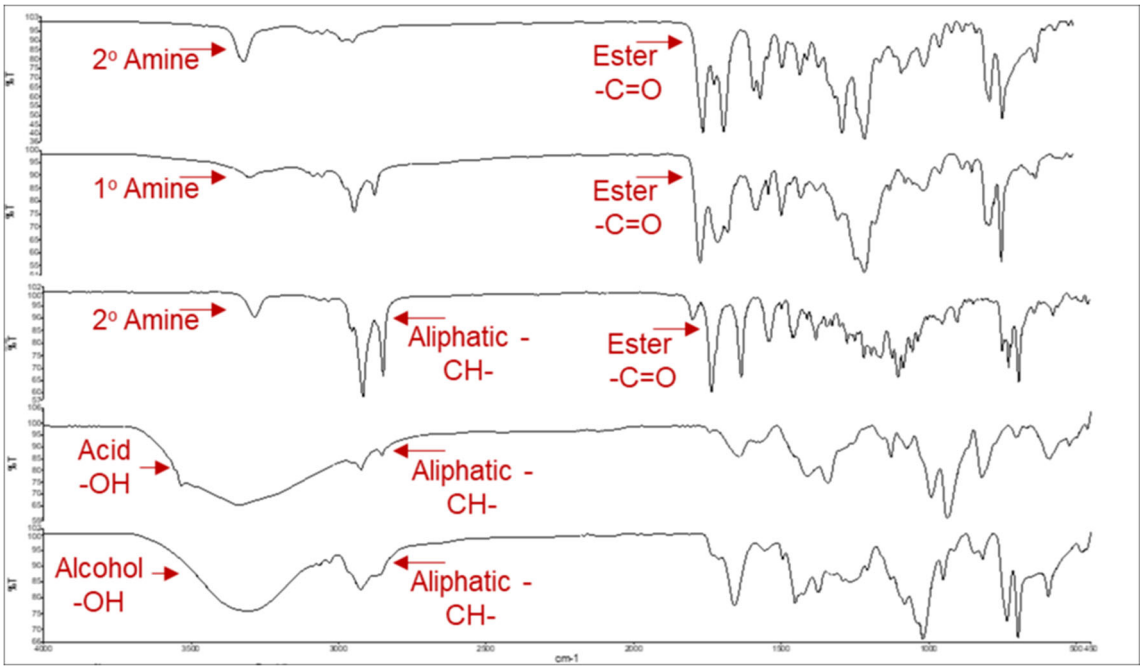


Fig. 2 Overlay of FTIR spectra of **1**, **2**, **3**, **4** and **5** (from top to bottom)

the rest of the structural similarities, compounds **1**, **2** and **3** demonstrated carbonyl peaks at $\nu_{max} \sim 1735\ cm^{-1}$ and $\nu_{max} \sim 1655\ cm^{-1}$ corresponding to carboxylic ester and amide functions, respectively. Similarly, the presence of benzyl groups was evident from the density of C-C stretching peaks between $\nu_{max} \sim 1600$ and $1450\ cm^{-1}$.

But disappearance of some of these features, i.e. carbonyl peaks of carboxylic ester and dense peaks of benzyl groups, confirmed reduction of terminal benzyl ester groups. Compound **3** was reduced under mild and vigorous reaction conditions to its corresponding carboxylic acid **4** and alcohol **5** analogues, respectively. Broad peaks at $\nu_{max} \sim 3350\ cm^{-1}$ and $\nu_{max} \sim 3302\ cm^{-1}$ were observed corresponding to hydroxyl groups of carboxylic acid and alcohol, respectively. The aliphatic C-H stretching at $\nu_{max} \sim 2950\ cm^{-1}$ as well as carbonyl peaks at $\nu_{max} \sim 1650\ cm^{-1}$ corresponding to carboxylic acid and amide functional groups was observed for both compounds **4** and **5**.

During mass analysis, all the synthesized compounds produced signals in positive $[M + H]^+$ as well as negative $[M - H]^-$ ionisation modes. The observed $[M + H]^+$ values, 616.43 and 560.74, for compounds **4** and **5** further confirmed not only reduction of compound **3** but also roles of respective reaction conditions [22]. Hence, analytical data confirmed structural details of the synthesized compounds.

Morphological surface characteristics of the synthesized compounds **3**, **4** and **5** were determined visually as well as using an optical microscope. Amongst the obtained solids, compound **3** was found to be crystalline, whereas compound **4** was found to be amorphous in nature. In contrast, compound **5** was isolated as a slightly viscous liquid.

Determination of Foaming Properties

Molecules with benzyl ester, acid and alcohol termini account for nonpolar neutral, polar anionic and polar neutral peripheries, respectively, due to their structural details. All synthesized molecules had hydrophilic and hydrophobic regions in some balance. Hence, foaming properties of the synthesized compounds **1**, **3**, **4** and **5** were determined to identify surfactant-like molecules. Foaming properties were observed to be in the order of **5** > **4** > **3**, **1**, blank (refer to Supplementary Information Fig. S13). Hence, as expected, molecules with polar termini showed better surfactant-like foaming properties compared with compounds with nonpolar termini. Moreover, amongst the polar termini molecules, stable foam was formed with compound with alcohol periphery. Whereas, irrespective to the presence of myristoyl chain, compounds with benzyl ester termini did not demonstrate any foaming nature. Instead, solutions with these compounds were turbid initially and eventually separated into precipitate. Although amide, ester as well as acid linkages impart polarity to synthesized dendritic lipopeptides, their contribution was found to be insufficient for imparting surfactant-like hydrophilic-lipophilic balance. In contrast, such a balance was established with considerable enhancement on replacement of peripheral benzyl groups with alcohol hydroxyls.

Determination of Polymeric Nanoparticle Forming Potential

Solvent evaporation method was used for preparation of polymeric nanoparticles. Particle size of formed nanoparticles was

determined by dynamic light scattering method. Average particle size of compound **3** was observed to be 293.5 nm with 0.131 polydispersity index (refer to Supplementary Information Fig. S14). Owing to its nonpolar nature, compound **3** formed polymer nanoparticles which remained suspended in solvent. Zeta potential of the same was found to be − 49.5 mV. In contrast, compound **4** and compound **5** were dissolved in water, without forming nanoparticles. Though zeta potential of resulting solution was found to be − 22.3 mV and − 26.6 mV, respectively. Hence, in the presence of water, compound **3** was demonstrated to self-assemble into nanoparticles with overall negative surface charge and narrow polydispersity index. Moreover, high magnitude of corresponding zeta potential indicates stability of formed suspension. Overall, amongst all the synthesized compounds, compound **3** demonstrated potential to form a stable nanosuspension.

Antibacterial Assay

SPL7013, a dendritic peptide molecule carrying negatively charged bulky peripheral groups, have been shown to possess inherent antimicrobial properties [23]. Our quest to identify any inherent antibacterial properties associated with the designed molecules was based on the aforementioned concept of SPL7013. Therefore, 1% w/v aqueous solutions of compounds **1**, **3**, **4** and **5** were tested against gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria using plate method of antimicrobial assay. Irrespective of their individual natures, none of the molecules demonstrated any inherent antibacterial activity (refer to Supplementary Information Fig. S15).

Anticancer Assay

Dendritic molecules with neutral or anionic surface charges were synthesized in order to develop compounds devoid of uncontrolled interactions with the cell membrane phosphate groups. Such an interaction is known to lead to cytotoxicity, which is favourable against cancer cells. To investigate any such inherently associated anticancer activity, despite absence of cationic surface groups, preliminary screening of compounds **3**, **4** and **5** was conducted at 10 μM concentration by MTT assay in MDA-MB-231 (breast cancer) cell line. In this study, compounds **3** and **4** demonstrated more than 70% cell viability (refer to Supplementary Information Fig. S16), indicating lack of any inherent anticancer activity. In contrast, compound **5** demonstrated less than 50% cell viability, indicating possible presence of anticancer activity due to the surface hydroxyl groups. However, its cytotoxic effect was significantly lower than the established anticancer drugs cisplatin and doxorubicin, which showed less than 25% cell viability. Hence, we conclude that the molecules with nonpolar neutral and polar anionic surfaces could not induce cytotoxicity, but molecule with polar neutral surface showed certain associated cytotoxicity. This information can be helpful while extending utilization of the synthesized compounds as excipient components of anticancer or other drug delivery systems, and hence deserve further safety investigation in detail.

Conclusion

Present study focuses upon development of neutral and/or anionic dendritic lipopeptide molecules, comprising L-glutamic acid dendrons and myristoyl chains. The multi-step solution phase reaction conditions adopted for present work were not only simplified but also fairly rapid. Moreover, molecules were designed to have benzyl ester, carboxylic acid and alcohol termini. These structural details accounted for nonpolar neutral (**3**), polar anionic (**4**) and polar neutral (**5**) peripheries, respectively. The synthesized nonpolar molecule **3** demonstrated potential to form self-assembled polymeric nanoparticles, whereas the polar molecules **4** and **5** demonstrated surfactant-like properties. Irrespective of their individual natures, none of the molecules demonstrated any inherent antibacterial activity against gram-positive as well as gram-negative bacterial strains. However, by virtue of surface hydroxyl groups, compound **5** demonstrated hints of anticancer activity in preliminary screening against MDA-MB-231. Hence, further safety investigation of these potential pharmaceutical materials would confirm the promising molecular candidates suitable for drug delivery applications.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Approval Not applicable

Consent to Participate Not applicable

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