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Solid-phase synthesis of arginine-based double-tailed cationic lipopeptides: potent nucleic acid carriers†

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Herein we report a highly-efficient solid-phase strategy for the modular synthesis of 63 double-tailed lipid-peptide conjugates and their application in DNA delivery.

Solid-phase organic synthesis is a versatile technique for the preparation of oligomeric compounds such as peptides[1] and peptidomimetics,[2] nucleic acids[3,4] and analogues,[5] oligosaccharides,[6] dendrimers[7] and small molecules.[8] Apart from the well-known advantages of solid-phase chemistry (*e.g.* forcing reactions to completion by mass action, simplified purification, compatibility with a broad range of solvents),[9] it enables splitting (and mixing when appropriately encoded)[5,10] during the stepwise incorporation of different building blocks.

Introducing DNA into cells, commonly termed transfection, has become an essential technique for countless biochemical studies and, in principle, can be used to treat both inherited and acquired diseases.[11] To facilitate DNA transfection, cell delivery vehicles have been developed, often based on liposomal formulations formed by self-assembly of cationic lipids.[11] To date, a wide range of cationic lipids has been generated and used as transfection reagents.[11–13] However, the determination of structure/transfection–activity relationships (STARs) remains elusive since the families of cationic lipids evaluated do not typically possess related structural features. In addition, transfection efficiency is often dependent on the targeted cell type and DNA size, while variations in media and DNA to transfection reagent ratios add additional complications. This makes prospective correlation efforts a rather erratic exercise.[11f–h] In the absence of defined structural criteria, a parallel approach can be beneficial to assist in the rapid determination of framework-specific STARs. To this end, parallel solid-phase synthesis is an optimal strategy for the systematic modification of the modular cationic lipid structure:[11f–h,12] (i) the cationic headgroup/s, (ii) the linking moiety, and (iii) the hydrophobic tail/s.

Herein we report a solid-phase strategy for the synthesis of 63 double-tailed lipid-peptide conjugates using a modular approach and their evaluation as DNA carriers (Fig. 1). Double-tailed cationic lipids were synthesised on a Rink amide-functionalised polystyrene resin,[14] which generates an amide group at the C-terminus upon resin cleavage, using the building blocks shown in Fig. 1. Encouraged by the remarkable aggregation properties displayed by gemini and gemini-like surfactants,[13] four trifunctional spacers containing symmetrical polyamines were used (Fig. 1, **SP1-4**). These were synthesised from diethylenetriamine and norspermidine using DdeOH to allow the selective blocking of the primary amines in the presence of the secondary amine, which could then be selectively coupled to either succinic or maleic anhydride (Scheme 1). These spacers were employed to investigate the optimal separation distance between the lipid tails and the role of spacer flexibility on the transfection abilities of the compounds.

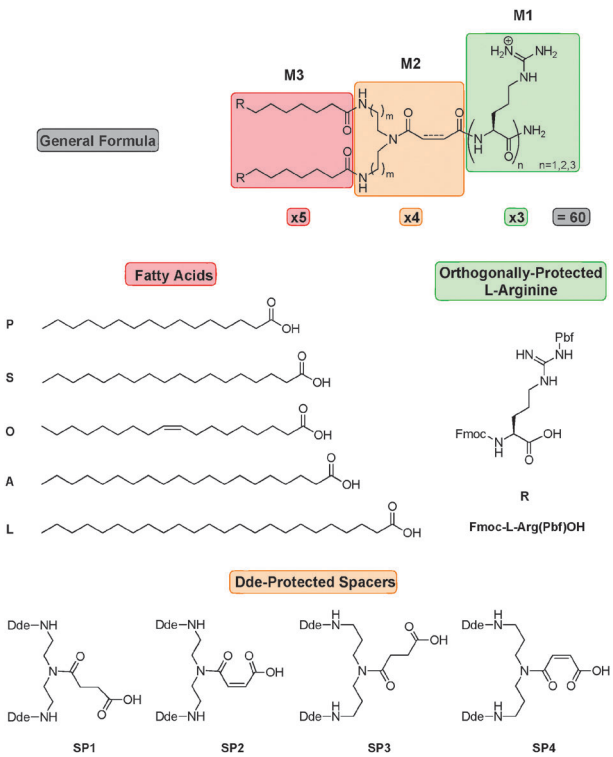

Fig. 1 General structure of the cationic lipopeptide library and the building blocks used in its synthesis (M1: the cationic headgroup; M2: the spacers and M3: the lipid tails).

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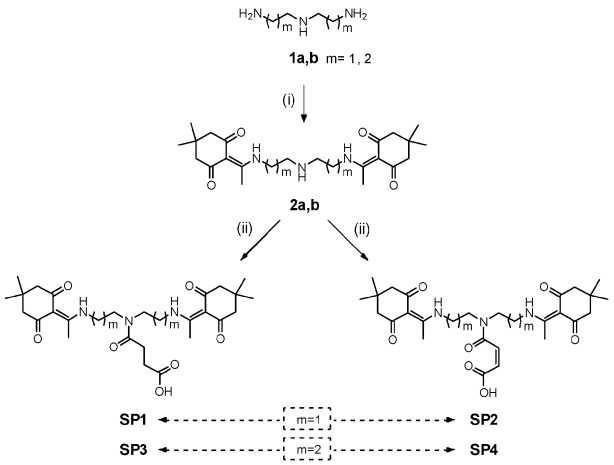
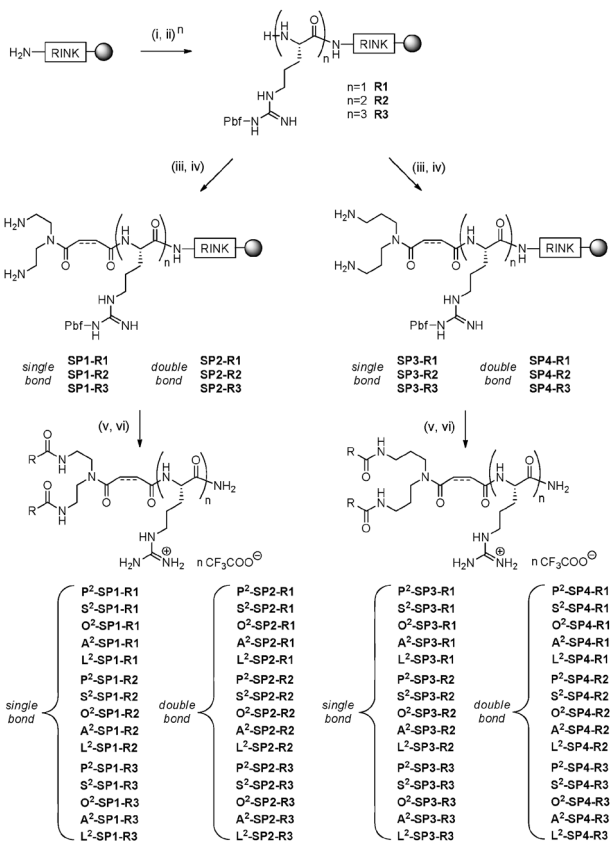
† Electronic supplementary information (ESI) available: Synthesis and compound characterization, transfection and cytotoxicity studies. See DOI: 10.1039/c1cc15805h



Scheme 1 Synthesis of Dde-protected spacers **SP1-4**. Reagents and conditions: (i) DdeOH (2.1 equiv.), DCM, 6 h; (ii) succinic or maleic anhydride (1 equiv.), DCM, 8 h, overall yield 91–94% (two steps).

The synthesis of 60 arginine-based lipopeptides was performed in parallel as described in Scheme 2 (for full description see ESI†). Microwave-assisted coupling of Fmoc-Arg(Pbf)-OH using DIC/HOBt was followed by Fmoc deprotection to give mono-arginine resin (**R1**). This protocol was repeated to give di- and tri-arginine scaffold resins (**R2** and **R3**). Resins **R1-3** were subsequently divided into four, independently coupled to the four Dde-protected spacers **SP1-SP4** and treated with 5% hydrazine in DMF to cleave the Dde groups.[14] The resins were further split into 5 and coupled to a set of fatty acids (palmitic (**P**), stearic (**S**), oleic (**O**), arachidic (**A**) and lignoceric (**L**) acid) using DIC/HOBt. Removal of the Pbf protecting groups and cleavage from the resin was achieved using a mixture of TFA/TIS/H_2O (95 : 2.5 : 2.5) to give the lipopeptides as their TFA salts.



Scheme 2 Solid-phase synthesis of arginine-containing double-tailed cationic lipids. Reagents and conditions: (i) Fmoc-Arg(Pbf)-OH (3 equiv.), DIC (3 equiv.), HOBt (3 equiv.), DMF/DCM (2 : 1), μW, 60 °C, 20 min; (ii) 20% piperidine in DMF, 10 min (×2); (iii) spacer (**SP1**, **SP2**, **SP3** or **SP4**, 3 equiv.), DIC (3 equiv.), HOBt (3 equiv.), DMF/DCM (2 : 1), μW, 60 °C, 20 min; (iv) 5% hydrazine in DMF, 2 h; (v) fatty acid (3 equiv.), DIC (3 equiv.), HOBt (3 equiv.), DMF/DCM (2 : 1), μW, 60 °C, 20 min (×2); (vi) TFA/TIS/H_2O (95 : 2.5 : 2.5), 2 h.

The DNA transfection abilities of the compounds were evaluated with HeLa cells employing pEGFP-C1 (4.7 kilobases) as a reporter. As shown in Fig. 2, EGFP expression results were strongly dependent on the number of arginine residues, with little transfection detected with the mono-arginine compounds. Overall, the presence or absence of unsaturation in the spacer moiety had a minor impact on transfection. Di-arginine and tri-arginine compounds with diethylenetriamine-based spacers (**SP1**, **SP2**) gave the highest transfection rates. In particular cationic lipids containing palmitoyl tails (**P**), the shortest fatty acid used in the library, and spacers **SP1** and **SP2** led to transfection rates comparable or even higher than Lipofectamine™ 2000. Flow cytometry analysis (Fig. 2b,d) confirmed that di-arginine compounds **P²-SP1-R2** and **P²-SP2-R2** gave the highest levels

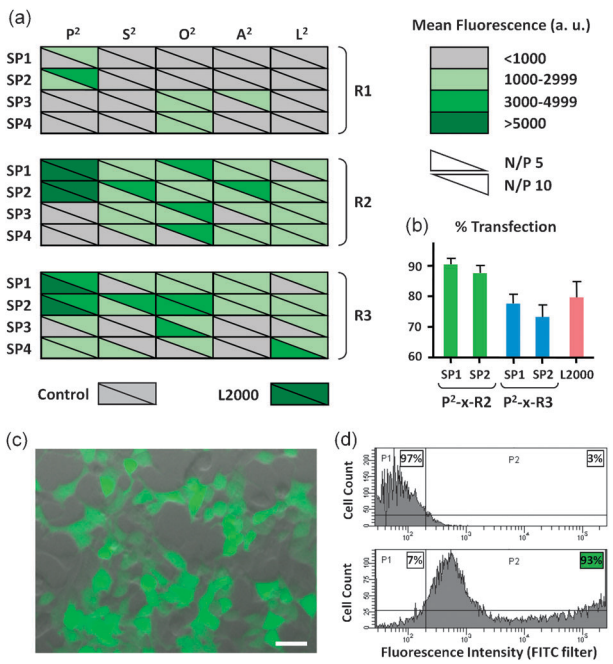


Fig. 2 Transfection screening of HeLa cells with arginine-based lipopeptides. Lipoplexes were formulated using DOPE as co-lipid (1 : 1 molar ratio) and pEGFP-C1 at N/P ratios of 5 : 1 and 10 : 1 and tested in triplicate. Lipofectamine™ 2000 (L2000) was used as the positive control and untreated cells as a negative control. (a) Mean fluorescence (a.u. = arbitrary units) of cell population 48 h after transfection (measured using a BioTek FLx800microplate reader: 485/20 excitation, 530/25 emission). (b) Percentage of transfected HeLa cells as measured by flow cytometry (FACSaria). (c) Merge image (brightfield + fluorescent channels) of HeLa cells transfected with **P²-SP1-R2**. Scale bar: 50 μm. (d) Flow cytometry histogram of untransfected HeLa cells (upper) and cells transfected with **P²-SP1-R2** (lower).

of transfection and MTT cell viability assays showed no cytotoxicity at the concentrations used.

Since **P^2-SP1-R2** (bearing C16 tails), showed superior transfection effiency compared to conjugates with longer fatty tails and the presence of double bond did not appear to be required, **SP1-R2** compounds with C14, C15 and C17 saturated fatty tails were synthesised as described above to investigate optimal lipid length. These compounds, along with the resynthesised hit **P^2-SP1-R2**, were evaluated against HeLa and HEK293T cells. Results showed that compound **P^2-SP1-R2** (C16) yielded the highest transfection levels (see ESI†), indicating that 2 arginines, a diethylenetriamine spacer and palmitoyl tails were optimal for DNA transfection with this class of cationic conjugates.

In conclusion, a straightforward and highly-efficient solid-phase strategy has been developed for the parallel synthesis of double-tailed lipid-peptide conjugates using a modular approach to rapid generation of comprehensive STARs as DNA carriers. This method was used to generate 63 lipo-arginines and allowed the identification of several highly efficient, non-toxic transfection reagents.

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