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Irregular cationic lipotetrapeptides for pharmaceutical multifunctional transport systems

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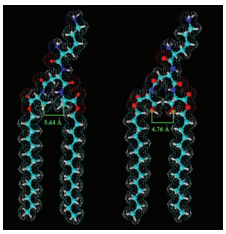
New cationic branched lipotetrapeptides based on L-amino acids and higher alkanols were prepared. Study of their physicochemical and membrane-forming properties revealed that phase transition temperature of these amphiphiles locates in the range of physiological norm. The particle sizes of liposomal dispersions with high aggregation stability are in dimension applicable to penetrate into small blood vessels.



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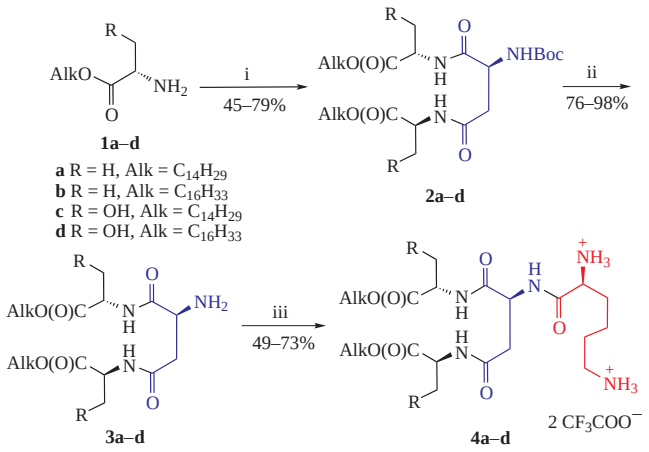
It is known that a number of extracellular and intracellular barriers are among the main problems on the path to the successful implementation of technologies based on micro- and nanoconstruction. These problems include premature degradation of active substances in the bloodstream because of the action of numerous nucleases, and immune response within a few minutes after intravenous injection.[1] Nowadays, the modern means to deliver biologically active compounds into cells make it possible to overcome such difficulties.[2,3] An interesting direction is the creation and investigation of cationic liposomes as non-viral vehicles. Such systems deliver therapeutic agents of various nature, as well as gene constructs, without causing a high toxic effect.[4]

Amino acids represent excellent materials for the synthesis of amphiphilic molecules[5,6] used in the pharmaceutical and biomedical research areas, where it is important to optimize the molecular safety profile. Generally, they have low cytotoxicity.[7] Their molecular structure allows one to vary the architecture of compounds to effectively control the properties of degradation and biological activity. In addition, the presence of chiral centers allows them to form aggregates with different morphology.[8] Also, amino acids are pH sensitive, which is a practically significant feature.[9]

Among natural amino acids, L-lysine, L-serine, and L-aspartic acid are of particular interest.[10,11] The structure of L-lysine provides its universal application as a building block in the synthesis of cationic amphiphiles: the presence of three active centers makes it possible to obtain molecules with different ionic characteristics.[12] Compounds containing L-lysine in the polar block are often used as mediators for transfection of genetic material into cells, as low-toxic non-viral delivery systems.[13] L-Aspartic acid is often used as a spacer to obtain a hydrophobic block.[14] L-Serine can reduce the overall lipophilicity of the molecule, making it safer.

The hydrophilic–lipophilic balance (HLB) is a characteristic of potential therapeutic molecules which determines fine details of their effectiveness. Calculations performed using the 'ACD / Labs, LogP' computer program showed that a range of HLB values of 11–15 (Table 1) corresponds to that of substances with high transfection activity.[15] These data became the basis for the development of schemes for the synthesis of target compounds.

In this work, we carried out the structural design and synthesis of new irregular cationic lipotetrapeptides (Scheme 1) based on L-Ser and L-Ala to determine the effect of the structure of the side functional group in the linker on the general physicochemical properties of liposomal aggregates based on them. The presence of a free hydroxyl group in L-Ser provides the advantage of reducing cytotoxic effects compared to analogues that do not contain a residue of this amino acid.[16] The structure containing L-Ala serves as a control for further biochemical studies. Higher C_{14} and C_{16} alkanols are employed in the hydrophobic block of the compounds. The polar block of amphiphiles is formed by the



Scheme 1 *Reagents and conditions*: i, BocAspOH, DCC, DMAP, CH_2Cl_2, 0 °C; ii, TFA, then 5% $NaHCO_3$; iii, Boc_2LysOH, DCC, DMAP, CH_2Cl_2, 0 °C, then TFA.

Table 1 The main characteristics of colloidal systems.

Compound	HLB	CPP	PTT/°C	Average size/nm	Polydispersity index	Zeta-potential/mV
4a	12.49 ± 0.69	0.78	34 ± 0.5	112.14 ± 11	0.181	39.71 ± 4.9
4b	14.61 ± 0.69	0.78	38 ± 0.5	235.0 ± 15	0.283	42.50 ± 3.7
4c	11.07 ± 0.71	0.77	30 ± 0.5	82.94 ± 9	0.224	31.09 ± 5.1
4d	13.20 ± 0.71	0.77	33 ± 0.5	146.90 ± 5	0.722	35.94 ± 4.9

L-Lys residue, while the spacer is L-Asp bearing two carboxy groups to form a hydrophobic domain.

The target compounds were constructed using the proven peptide synthesis techniques.[17,18] Esters **1a–d** were obtained by the reactions of L-Ala or L-Ser with an excess of aliphatic alcohols $C_{14}H_{29}OH$ or $C_{16}H_{33}OH$. Compounds **2a–d** were obtained by the conjugation of a Boc-derivative of L-aspartic acid with hydrophobic blocks **1a–d**, respectively, employing the Steglich DCC/DMAP system. The deprotection of Boc-derivatives **2a–d** with TFA gave trifluoroacetate salts which were neutralized with $NaHCO_3$ (aq.) to afford compounds **3a–d**. The final cationic amphiphiles **4a–d** were prepared by reacting compounds **3a–d** with Boc_2LysOH using DCC/DMAP system followed by deprotection. The structures of target products were characterized by NMR spectroscopy and mass spectrometry.†

Critical packing parameter (CPP) calculations with the 'ACD / Labs, 3D Viewer' program showed that the synthesized compounds form bilayer spherical aggregates in the aquatic environment (see Table 1).[19] A thin lipid film was hydrated with distilled water, followed by sonication, to prepare liposomal dispersions.[20]

The phase transition process for bilayer aggregates was investigated using a method based on the binding of amphiphilic molecules to the eosin dye. In an aqueous solution, the dye exists as a monomeric and dimeric form. A linear transition of the dye from a dimeric form to a monomeric one occurs with increasing the temperature. In the presence of liposomes, the dependence of monomer–dimer concentrations on temperature is not linear, and when the temperature of the phase transition temperature (PTT) of the lipid is reached, the line of dependence of the optical absorption intensity on temperature is bent.[21] In this way, PTT values for compounds **4a–d** were determined (see Table 1).

Particle sizes were estimated for all dispersions by dynamic laser light scattering (DLS, see Table 1). Experiments show that the presence of the L-Ser hydroxy group in amphiphiles **4c,d** (Figure 1) reduces the average size of the formed aggregates, as compared to L-Ala analogues **4a,b**. This could be indicative of some hydrogen bonding between the hydroxy groups of L-Ser

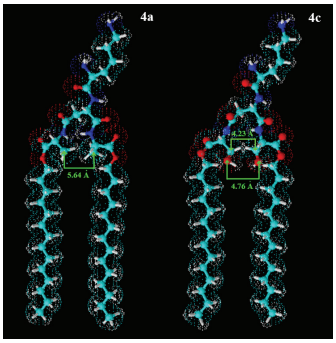


Figure 1 3D structures of compounds **4a** and **4c**.

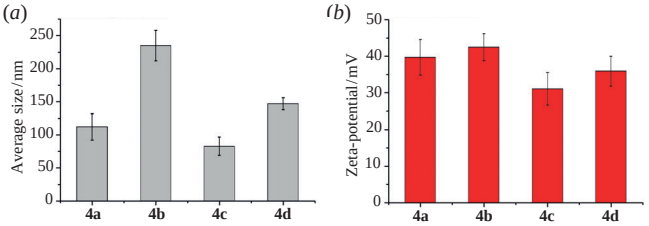


Figure 2 (*a*) The average size and (*b*) the zeta-potential of liposomes **4a–d**.

occurring in the corresponding liposomes. Elongation of the aliphatic chain of the hydrophobic block enhances this effect [Figure 2(*a*)].

It gives an idea to suppose the possibility and to evaluate the speed of the relative movement of the dispersed phase and the dispersion surrounding, the intensity of electrokinetic phenomena, the stability of sols and the destruction of the dispersed system by electrolytes. For all compounds the zeta-potential has high values (see Table 1). Due to this, electrostatic repulsion of particles in an aqueous environment occurs, which serves as an explanation for the high stability of colloidal solutions for a long storage time at room temperature (more than two weeks). The presence of the L-serine hydroxy group in compounds **4c,d** reduces their zeta-potentials, but not crucially to affect the stability of the systems.

In summary, we synthesized new cationic amphiphiles based on natural L-amino acids. The synthetic protocol is simple, versatile and scalable. The studies of liposome dispersions in aqueous solutions showed that the presence of hydroxy group in L-Ser residue allows one to obtain dispersed particles diameter 20 nm smaller in comparison with structures containing L-Ala. The values of the zeta-potential for the formed aggregates do not depend on the length of the hydrocarbon chain and are within the universal range (30–40 mV) for transfection mediators.[22] The phase transition temperatures of the components of cationic liposomes lie within the physiological norm, which confirms their further applicability in *in vitro* experiments.

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† *Synthesis of lipotetrapeptides.*

To a solution of BocAsp (0.36 g, 1.55 mmol) in CH_2Cl_2 (10 ml) and DMF (350 μl) cooled to 0 °C, DMAP (0.57 g, 4.65 mmol), DCC (1.28 g, 6.21 mmol) in CH_2Cl_2 (10 ml) and amino ester **1a** (1.11 g, 3.88 mmol) in CH_2Cl_2 (20 ml) were added. The mixture was vigorously stirred for 24 h. Final column chromatography afforded 0.73 g (61%) of compound **2a**.

To a solution of compound **2a** (0.56 g, 0.73 mmol) in CH_2Cl_2 (25 ml), a solution of TFA in CH_2Cl_2 (12 ml, 1:1 v/v) was added at 0 °C, and this was stirred at 0 °C for 2 h. After the reaction was complete, the volatiles were distilled off *in vacuo*. The residue was dissolved in $CHCl_3$ (15 ml), and the solution was sequentially washed with 10% $NaHCO_3$ (4 × 15 ml) and water to pH 7, dried over Na_2SO_4, and the solvent was distilled off. The yield of product **3a** was 0.52 g (92%).

To a solution of Boc_2LysOH (0.40 g, 1.16 mmol) in CH_2Cl_2 (15 ml) cooled to 0 °C, DMAP (0.28 g, 2.32 mmol), a solution of DCC (0.48 g, 2.32 mmol) in CH_2Cl_2 (20 ml), and a solution of compound **3a** (0.51 g, 0.77 mmol) in CH_2Cl_2 (20 ml) were added. The mixture was vigorously stirred at 0 °C for 4 h and then at room temperature for 24 h. The product was isolated by column chromatography. The yield of compound **4a** after Boc-deprotection was 0.46 g (60%). Compounds **4b–d** were synthesized similarly. For their characteristics, see Online Supplementary Materials.

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2021.07.024.

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