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Solid-phase synthesis and purification of a set of uniformly ^{13}C, ^{15}N labelled *de novo* designed membrane fusogenic peptides

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Abstract: The transmembrane segments of soluble *N*-ethylmaleimide-sensitive factor (SNARE) proteins or viral envelope proteins drive membrane fusion, which suggests that simple synthetic biology constructs for fusion exist and can be evaluated. We describe the high-yield synthesis of a set of *de novo* designed fusogenic peptides for use in functional investigations, which are highly enriched in ^{13}C and ^{15}N using three equivalents of labelled amino acids and optimized reaction conditions minimizing aggregation. The biomimetic peptides have a high purity >90% and show reproducible and fusogenic activity that correlates well with the intended functional design characteristics, from strongly fusogenic to almost non-fusogenic. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: solid-phase peptide synthesis and purification; isotope labelling; membrane fusion

INTRODUCTION

Synaptic transmission is initiated when an action potential triggers neurotransmitter release from a presynaptic nerve terminal [1]. All the presynaptic functions, directly or indirectly, involve synaptic vesicles. Synaptic vesicles undergo a trafficking cycle in the nerve terminal and deliver their load [1]. A major goal in neurobiology in recent years has been to gain insight into the molecular machinery that mediates neurotransmitter release. More than 1000 proteins function in the presynaptic nerve terminal and hundreds are thought to participate in exocytosis [1]. In this protein zoo, which proteins are actually important and which are only bystanders is difficult to answer. However, the integral membrane proteins such as soluble SNAREs are essential for fusion of cargo vesicles [2,3]. A direct role of their transmembrane segments in fusion is supported by recent *in vitro* studies [4–7]. For instance, TMDs of presynaptic SNAREs or of the VSV G-protein, represented by synthetic peptides, were shown to drive fusion of liposomal membranes. In addition, evidence has been obtained that transmembrane segments of fusogenic membrane proteins display conformational flexibility and that this enhances fusogenicity [7–9]. This has encouraged us to mimic these transmembrane segments with *de novo* designed synthetic peptides [8]. When the structure of these transmembrane peptides was analysed in an isotropic solution by CD spectroscopy, we found that mutations that stabilize α-helicity of the peptides and disfavour β-sheet formation tend to compromise their fusogenicity. In addition, it was proposed that sheet formation by the fusogenic peptides *in vitro* reflects a structural flexibility of the original helices, which is rather unusual for transmembrane segments of other membrane proteins and may be essential for their fusogenicity [9].

For decades, the question of how a protein's amino acid sequence dictates its conformation has captivated the attention of many chemists, biophysicists, and biochemists. In the past, we have assessed whether SNARE transmembrane segments exhibit an unusual amino acid composition by comparing the average amino acid compositions of the TMDs of different isoforms of synaptobrevin and syntaxin to that of a large database of unrelated TMDs (TMbase 25 [10]) [9]. An analysis of amino acid composition revealed that isoleucine and valine are strongly present in transmembrane segments from different SNARE subtypes and orthologues as compared to a large database of unrelated

Abbreviations: DOPS, 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine]; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; HBTU, [2-(1 H-benzotriazol-1-yl)-1,1,3,3-etramethyluronium hexafuorophosphate]; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)hexadecylphosphatidyl-ethanolamine; NMP, *N*-methylpyrrolidinone; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; Rh-PE, *N*-(lissamine rhodamin B sulfonyl)hexadecylphosphatidyl-ethanolamine; SNARE, soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment protein receptor; TMD, transmembrane domain; TIS, triisopropyl silane.

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transmembrane segments [9]. Isoleucine and valine rank among those residues with the highest propensities to form β-sheet structures [11–13]. This property was ascribed to steric interference of their β-branched side chain with the local polypeptide backbone that may destabilize the α-helical conformation. Our studies show that the fusogenic peptides with mixed Leu and Val sequences in the hydrophobic core exhibit a higher degree of conformational plasticity than non-fusogenic peptides with oligo-Leu or oligo-Val cores [9]. Since fusogenicity correlates with structural flexibility for this class of peptides, the dynamics of the secondary structure apparently controls membrane fusogenic properties [8]. The helix-destabilizing residues enhance fusion protein function via transmembrane conformational dynamics, and the structural rationale behind this is that Gly does not support the packing of side chains along a helix backbone, whereas Pro cannot form a hydrogen bond to the i-4 residue [8]. Both residues cause destabilization of transmembrane helices in membrane-mimetic environments and organic solvents [14,15]. Pro residues in transmembrane helices are frequently associated with local kinks, especially when a Gly residue is present in the vicinity, and it has been argued that these kinks may function as 'molecular hinges', i.e. sites of increased local flexibility [16]. These non-natural polymers present a new system that provides an excellent medium for the design of biomimetic structures with practical applications in the areas of pharmaceuticals and materials science.

Although the structural flexibility of our fusogenic LV variants manifested itself as helix-to-sheet transition in an isotropic solution, how this transition takes place in the low dielectric environment of a membrane is still unclear. The behaviour of lipids in presence of these peptides observed by using ^{31}P solid-state NMR shows drastic changes and rearrangements of lipid phases and homogenization of lipid molecules across the lipid bilayer to mediate fusion (Agrawal *et al.* submitted). The corresponding phase changes may be related to the conformationally flexible behaviour of these peptides, in a sense that they locally unwind in the bilayer and impose a structural frustration facilitating lipid mixing. The interplay of flexibility and other features such as hydrophobic length and the nature of flanking residues with lipids will have to be studied in the future for a more complete understanding of membrane fusion.

To pave the way for an in-depth analysis of these phenomena by NMR spectroscopy, we proceed here with the preparation of a set of uniformly labelled peptides that show different fusogenic activities. This article describes the high-yield synthesis and purification of a *de novo* designed set of synthetic hydrophobic peptides containing uniformly ^{13}C, ^{15}N isotopically labelled 16-residue cores. These peptide hydrophobic cores mimic transmembrane sequences in the sense that they contain helix- or sheet-promoting residues. Such peptides will tend to aggregate owing to an inter-chain association involving hydrogen bonding between the secondary amide groups of the peptide chain, which leads to β-sheet formation, and helix-destabilizing residues like Gly and Pro are introduced in the middle of the core to hinder aggregation. The chosen peptides have 23 residues consisting of a central (Leu and/or Val)$_n$ repeating unit with $n = 8$ or 16. This unit is flanked on both sides by 3 Lys to enhance peptide incorporation in lipid bilayers and contains a Trp residue at one end for quantification. The sequences of these peptides are written below and the amino acids, which are underlined, are uniformly labelled.

L16: K K K W L L L L L L L L L L L L L L L K K K

LV16: K K K W L V L V L V L V L V L V L V L V K K K

LV16G$_8$P$_9$: K K K W L V L V L V L G P V L V L V L V

K K K

The peptides are synthesized using Fmoc solid-phase peptide synthesis (SPPS). Fmoc SPPS is a rapid, efficient, and reliable method for the synthesis of peptides and proteins [17,18]. In addition, for NMR spectroscopic studies on peptides in biomimetic lipid bilayers, they have to be isotopically labelled in both ^{13}C and ^{15}N to enhance the signals, and this puts additional constraints on the possible synthetic routes and purification. We have optimized the conditions used for the synthesis to give favor aggregation problems and better yield as compared to standard SPPS conditions to pave the way for isotope labelling. The crude peptides are purified using modified RP-HPLC methods. This gives high cost efficiency for the very expensive uniform labelling of these peptides. Finally, the purified peptides give us reproducible and reliable fusogenic assay data sets, opening up a novel route for obtaining valuable insight into the complex world of membrane fusion.

MATERIALS AND METHODS

All reagents were purchased from Acros Chemicals and used without further purification unless otherwise stated. Solvents and reagents used in the peptide synthesis were all of peptide synthesis grade and were purchased from Biosolve, except for HATU, which was from Applied Biosystems. TIS was supplied by Aldrich Chemical Co. All amino acids used were the naturally occurring L-amino acids. Fmoc-Lys(Boc) preloaded TentaGel S resin was bought from Rapp Polymere. The two isotope-labelled Fmoc protected amino acids, Fmoc-[U-^{13}C, ^{15}N]-Leu-OH and Fmoc-[U-^{13}C, ^{15}N]-Val-OH, were purchased from Cambridge Isotope Laboratories. Fmoc protection of [U-^{13}C,^{15}N]-Pro-OH, and [U-^{13}C,^{15}N]-Gly-OH was carried out as described [18,19]. Synthesis of the peptides was performed on an automated CS336 (CS Bio, US) peptide synthesizer. Finally, MALDI-TOF spectra of the purified peptides were recorded using on a Voyager-DE PRO mass spectrometer

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(Perseptive Biosystems, Inc.). POPC lipid was purified from fresh hen egg yolk; DOPE and DOPS were purchased form Avanti Polar Lipids, US. The liposome–liposome fusion assay was measured at a temperature of 37 °C.

SYNTHESIS OF THE PEPTIDES

Selected uniformly ^{13}C, ^{15}N-labelled peptides were prepared via the solid-phase Fmoc method on a commercially available preloaded Fmoc-Lys(Boc) TentaGel S PHB resin (265 mg, 0.25 mmol/g NH_2). The consecutive steps in the solid phase peptide synthesis performed in each cycle were: (i) Deprotection of the Fmoc-group with 20% piperidine in 20% DMSO/NMP for 5×2 min. (ii) Coupling by applying a three-fold excess of the appropriate amino acid. The amino acid (0.25 mmol) and HATU (0.225 mmol) were dissolved in 20% DMSO/NMP (1 ml) and subsequently 0.5 ml of 1 M DIPEA in NMP was added. After 1 min preactivation time, the mixture was transferred to the reaction vessel, which was shaken for 2 h. (iii) Capping the unreacted amino functions were by acetylation with 0.5 M acetic anhydride and 0.125 M DIPEA in NMP. (iv) Obtaining the free peptides were after treatment of the resin with a mixture of 95% TFA/2.5% TIS/2.5% H_2O. The synthesized peptide was filtered in cold ether and the precipitate was collected after centrifugation.

PURIFICATION OF THE PEPTIDES

Purifications were conducted on a BioCAD Vision' automated HPLC system (PerSeptive Biosystems, inc.), supplied with semipreparative Alltima (Alltech) columns (250 mm × 10 mm, running at 4 ml/min). The buffer gradient used for purification contained, A: 25% methanol + 75% water, B: 100% CH_3CN, and C: 1% TFA + 99% methanol. Linear gradients of B in 10% C were applied over 3 CV unless stated otherwise. The purification of peptides LV16 and L16 was performed with an Alltima CN phase column, and for LV16G8P9 a Prosphere C_{18} column was used. Detection was by UV spectroscopy at 214 and 254 nm. The final peptide purity was confirmed by MALDI-TOF. The peptides were lyophilized from a 10% acetic acid and water mixture. The lyophilized peptides were stored at −20 °C until used for the fusion assays.

Preparation of Small Unilamellar Liposomes

Liposomes were prepared from mixtures of egg PC/brain PE/brain PS at a ratio of 3 : 1:1 (w/w/w) with or without 0.8% (w/w) of NBD-PE and Rh-PE (Molecular Probes) [9]. Lipid solutions in chloroform with or without transmembrane peptides were first dissolved in TFE, dried under a stream of nitrogen as a thin film, and rehydrated with a fusion buffer (25 mM tris-HCl, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 5 mM DTT). Liposomes were formed using sonication (Bransonic Sonifier W450 cup horn) for 8 min and cooling with ice. The sample was centrifuged at 16 000 × g for 20 min to remove lipid aggregates. Peptide/lipid ratios were determined after separating peptides from proteoliposomes by density gradient centrifugation [6]. The liposomes were lysed with 1% (wt/vol) SDS and the amounts of lipid-associated peptide were determined using the Trp fluorescence at $\lambda_{ex} = 290$ nm of the top fractions.

Fusion Assays

Fusion assays were performed using the fluorescence dequenching method [9]. Briefly, 'donor' liposomes containing fluorescence labels and unlabelled 'acceptor' liposomes (2.5 mg/ml phospholipid) were mixed in the ratio of 1 : 4 (v/v) on ice, transferred to 96-well white Corning non-binding plates with translucent bottoms, and heated for 2 min by floating the plates on a 37 °C water bath. NBD fluorescence was immediately assayed at 1 min intervals for 60 min at 37 °C. Initial rates of fusion were obtained by fitting the first 10 min of the kinetics using ORIGIN software (OriginLab Corporation, Northampton, MA) with a polynomial function and determining its first derivative. All values were corrected for detergent quenching that was below 4%. The peptide-independent, spontaneous fusion of pure liposomes was routinely determined in parallel and subtracted from the values obtained with peptide-containing liposomes.

RESULTS

The MALDI-TOF spectrum of L16 ($M + H^+ = 2895$), with a uniformly labelled inner 16-residue core (Figure 1) shows a peak at m/z value of 2896.23 and other peaks with sodium (Na)/potassium (K) clusters 2918.25 $(M + Na)^+$, 2935 $(M + K)^+$, and 2940 $(M + 2Na)^{2+}$. Similarly, $LV16G_8P_9$ ($M + H^+ = 2715$), with a uniformly labelled inner 16-residue core (Figure 2) gave a major peak at m/z value of 2714.76 and other peaks with sodium (Na)/potassium (K) clusters 2736.65 $(M + Na)^+$, 2753.41 $(M + K)^+$, and 2758.64 $(M + 2Na)^{2+}$. In addition to these major peaks, there are some small peaks present that are difficult to separate, which makes these peptides >90% pure. Peptide LV16 ($M + H^+ = 2775$), with a uniformly labelled inner 16-residue core (Figure 3) gave two clusters of peaks. The major peak at m/z values of 2776.21 and other peaks associated with these peak are sodium (Na)/potassium (K) clusters 2796.84 $(M + Na)^+$ and 2820.27 $(M + 2Na)^{2+}$. The other minor cluster of peaks is from the fragment with an amino acid short, 2670.88 (LV16-K) and its sodium

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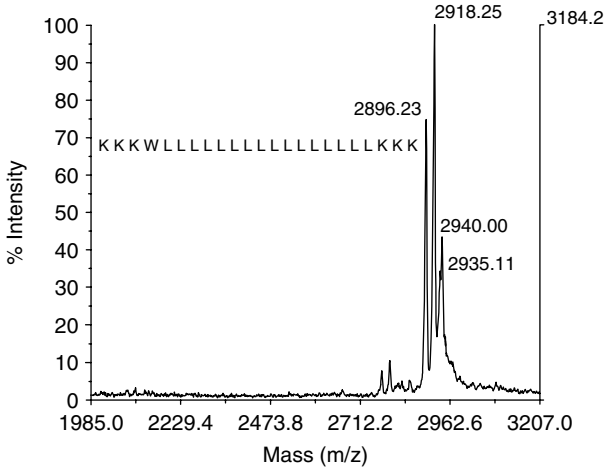

Figure 1 MALDI-TOF spectrum of the peptide **L16** ($M + H^+ = 2895$), with uniformly labelled inner 16-residue core, obtained after purification. Beside the main peak at 2896.23, there are sodium and potassium clusters of the main peak present.

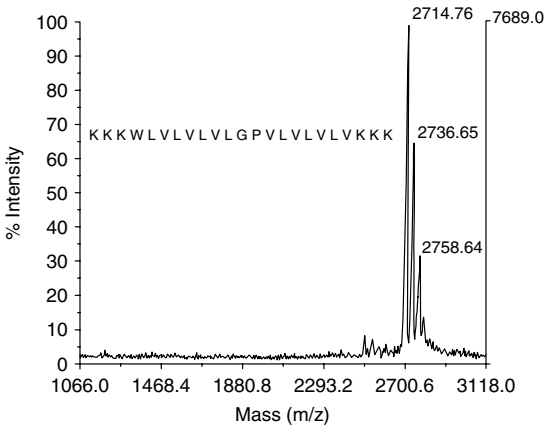

Figure 2 MALDI-TOF spectrum of the peptide **LV16G$_8$P$_9$** ($M + H^+ = 2715$), with uniformly labelled inner 16-residue core, obtained after purification. Beside the main peak at 2714.96, there are sodium and potassium clusters of the main peak present.

cluster 2692.47. In addition, there are some small peaks of the fragmented peptide that are difficult to separate using the modified purification method. The purity in this case is about 90%.

The peptides were incorporated into liposomal membranes by sonication at different peptide/lipid (P/L) ratios. Liposome–liposome fusion was examined by a standard fluorescence-dequenching assay upon rapidly shifting the temperature to 37°C. This assay is based upon fluorescent resonance energy transfer from NBD-PE to *N*-(lissamine rhodamin B sulfonyl)hexadecylphosphatidyl-ethanolamine (Rh-PE), being present at quenching concentrations in 'donor' liposomes. Upon fusion of labelled liposomes with unlabeled liposomes, which hold the peptides, the average distance between the fluorophores, and thus NBD

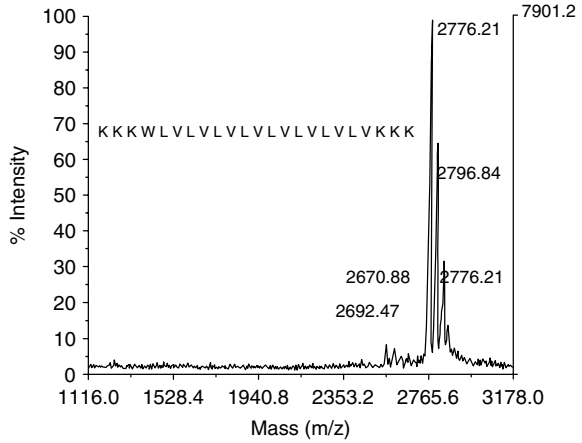

Figure 3 MALDI-TOF spectrum of the peptide **LV16** ($M + H^+ = 2775$), with uniformly labelled inner 16-residue core, obtained after purification. Beside the main peak at 2776.21, there is another peak at 2670.88, which is assigned to the one amino acid (Lys) short impurity that is difficult to separate. The clusters of sodium and potassium of main and side peak are present.

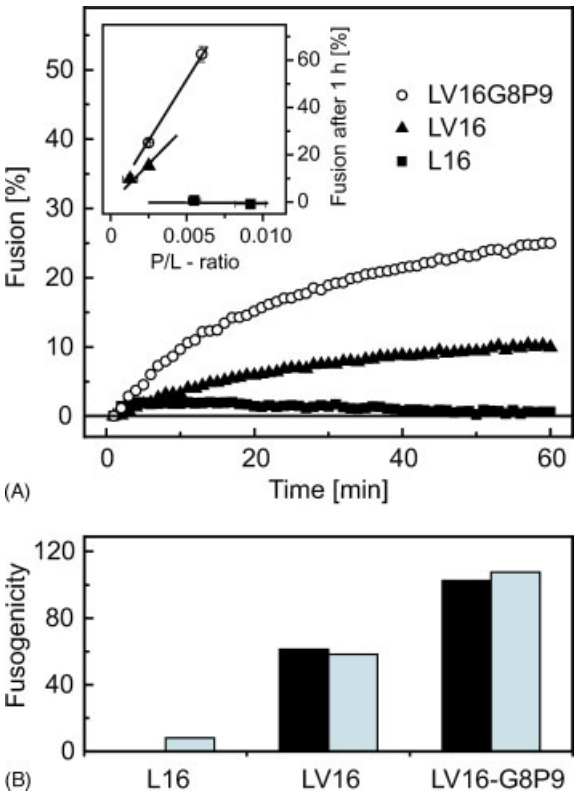

Figure 4 Fusogenicity of labelled peptides. **(A)** Original fusion kinetics of **L16**, **LV16**, and **LV16 – G$_8$P$_9$** ($n = 6$). The values were corrected for spontaneous fusion of liposomes without peptide, as measured in parallel. The incorporation of these peptides in liposomes was determined experimentally by measuring the P/L ratios. These values were correlated with the fusion extents seen after 1 h of incubation (Inset); note that LV16 incorporated somewhat less efficiently than L16 or LV16G8P9. (B) Labelled peptides (black bars) show similar fusogenicity like unlabeled peptides (gray bars [8]).

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fluorescence, increases over time; this is taken as a measure of lipid mixing. Fusion kinetics of peptides is compared in Figure 4, which indicates membrane fusogenicity of these peptides. These values were correlated with the fusion extents seen after 1 h of incubation.

DISCUSSION

The incorporation of synthetic peptides representing the transmembrane segments of membrane fusion proteins into the membranes of synaptic liposomes strongly increases their ability to fuse [8]. This *in vitro* fusion system consisting of only lipids and transmembrane peptides appears to display characteristic hallmarks of biological membrane fusion, which is a complex process. The structure–function correlation studies on these peptides help in understanding the complexity of this process. The peptides in the chosen *de novo* set are hydrophobic sequences with the repetitive units of amino acid residues, which tend to aggregate. Aggregation of hydrophobic peptides on resins generally leads to low coupling yields and an accumulation of single amino acid deletions, which are difficult to separate from the target peptide [18,20]. During Fmoc SPPS, aggregation through inter-chain association of the growing resin-bound peptides is known to contribute to the difficulty of synthesis [21,22]. Aggregation typically results in a decrease in rates of acylation and deprotection, and consequently leads to the production of deletion products of varying lengths and composition. Such a decrease in the purity of the crude material often results in subsequent difficulty in purification. To overcome the difficulties of decreased coupling efficiency, low yield, and aggregation, we implement a modified Fmoc SPPS method. The onset of internal aggregation in SPPS is usually indicated by slow acylation and/or by shrinkage of the swollen gel resin. We applied TentaGel S resin with low loading (0. 26 mmol/g), which is known to provide the best conditions for reducing aggregation [18].

To synthesize these hydrophobic peptides using conventional SPPS (PyBOP/DIPEA) and double coupling requires 10 equivalents of amino acids per coupling step (twice 5 eq.). Out of these 10 equivalents used, 9 are wasted, which is unacceptable for isotope labelling. To overcome this hurdle, a strong activator, increased coupling time, and different solvent mixtures are used. In particular, HATU reagent does not require double coupling and thus allows us to reduce the consumption of Fmoc amino acids in the synthesis of hydrophobic peptides [23,24]. The use of HATU doubles the yield of the peptide as compared with PyBOP or HBTU and eliminates double couplings. In this way, we have decreased the consumption of uniformly labelled amino acids to only 5 equivalents at each coupling step.

The advantages of DMSO as a valuable disaggregating solvent in SPPS have been known for some time, both as a neat coupling solvent and mixed with polar (e.g. DMF, NMP) and non-polar (e.g. THF, toluene) co-solvents [25–29]. In addition, DMSO is a powerful co-solvent for the dissolution of poorly soluble amino acid derivatives and reagents. By using a solvent mixture containing 20% DMSO and increasing the coupling time from 1 h to 2 h, the consumption of amino acids was further reduced to 3 equivalents at each coupling step. This implies that only 2 equivalents of amino acids are wasted, allowing the synthesis of uniformly labelled peptides in a cost-effective manner.

Previous experience using unlabelled commercial peptides has shown that the purity of these peptides plays an important role in obtaining reproducible and consistent data sets. However, hydrophobic peptides have a tendency to stick to the standard RP columns like C_8 or C_{18}, particularly if elution is performed with the standard acetonitrile–water system. This generally leads to increased backpressure and low yields of the purified peptides. Therefore, to purify these hydrophobic peptides to a high level is a major challenge. Purification of L16 and LV16 peptides is found to be most efficient and reproducible using a CN phase polar column, while for the $LV16G_8P_9$, a C_{18} wide pore column is sufficient. It proved to be essential to include 25% methanol in the HPLC buffer to eliminate the backpressure problem. This purification method gives both high purities and high yields (Table 1).

To test whether the synthesized peptides give reproducible and consistent fusion in liposomes, we have analysed the fusion kinetics of L16, LV16, and $LV16G_8P_9$ peptides that were synthesized and purified under the same conditions and were not isotope-labelled (Figure 4). Although the LV16 sample contains minor impurities, this does not change its fusogenic activity. In line with earlier results using peptides synthesized by Boc-chemistry, the assays show that L16 is the least fusogenic and $LV16G_8P_9$ is the most

Table 1 Purification and characterization of transmembrane peptides prepared in this study. Different solvent systems and gradient profiles used for the respective columns are mentioned in detail in text and purity is determined by using both HPLC and MS

	L16	LV16	$LV16G_8P_9$
Column	C4	CN	C18
Gradient	15–90 Acetonitrile	10–90 Acetonitrile	20–90 Acetonitrile
Yield	4.15 μmol	4.32 μmol	4.79 μmol

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fusogenic peptide, while LV16 shows intermediate fusogenic activity [8].

CONCLUSIONS

The cost-effective strategy described in this article for these *de novo* designed peptides is efficient and promising for the synthesis and purification of other hydrophobic peptides. The functionality of these peptides paves the way for detailed structural and dynamic interactions studies of these uniformly labelled peptides in lipid bilayers and also the effects of these peptides on lipid behaviour. In the future, spectroscopic studies on these labelled peptides, incorporated in lipid bilayers, will also contribute to the elucidation of the mechanism by which these peptides fuse lipid bilayers and thus will provide valuable information on membrane fusion.

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