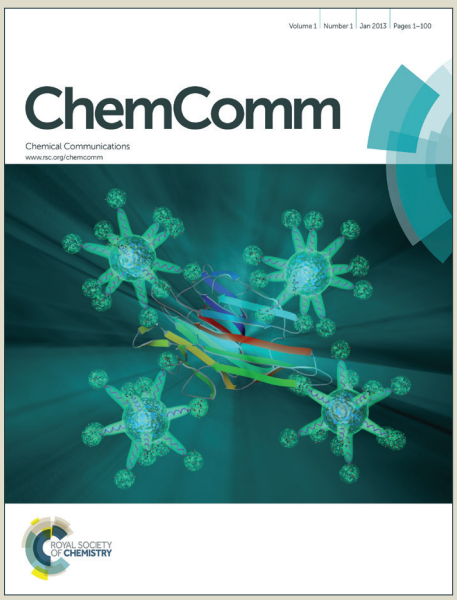
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

COMMUNICATION

Unsaturated acyl chains dramatically enhanced cellular uptake by direct translocation of a minimalist oligo-arginine lipopeptide

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

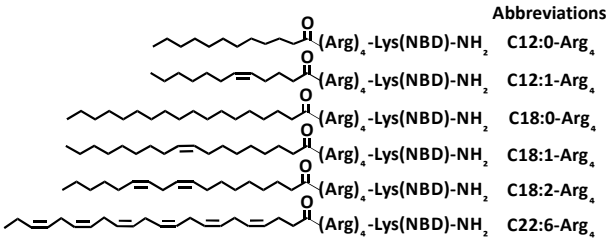
J.-M. Swiecicki,[a,b,c,†] M. Di Pisa,[a,b,c,†] F. Lippi,[a,b,c] S. Chwetzoff,[a,c,d,e,f] C. Mansuy,[a,b,c] G. Trugnan,[a,c,d,e] G. Chassaing,[a,b,c] S. Lavielle,[a,b,c,*] F. Burlina [a,b,c,*]

Abstract. The recurring issue with cell penetrating peptides is how to increase direct translocation *vs.* endocytosis, to avoid premature degradation. Acylation by a *cis* unsaturated chain (C22:6) of a short cationic peptide provides a new rational design to favour diffuse cytosolic and dense Golgi localisations.

Cell penetrating peptides (CPPs) can enter cells by energy-dependent (endocytosis) and energy-independent pathways (direct translocation).[1-7] The relative contribution of translocation, which depends on the CPP and on the cell line may account for 10 to 70% of the total internalisation.[8,9] The central but tricky question is how to increase direct translocation *vs.* endocytosis?

The key role of phospholipids (PLs) in the transfer of CPPs *via* the formation of inverted micelles has been proposed at the early stage of the CPP discovery,[10] and later extended to the "mutual adaptation" concept.[7] We recently demonstrated that cationic CPPs recruit anionic PLs on the outer leaflet of large unilamellar vesicles (LUVs), in the fluid phase, and consequently diffuse through the bilayer as a neutral lipophilic CCP/PL complex.[11-13] CPPs may also translocate into LUVs presenting fluid domains in gel phase or LUVs made of ternary mixture including cholesterol.[14] All these data led us to propose that direct translocation might be enhanced if CPPs could target and/or induce disordered domains into cell membrane.[15] Acylation of the CPP by an unsaturated fatty acid might be a key feature for that purpose. Acylation by a saturated chain of linear or cyclic CPPs has been previously shown to increase cellular internalisation,[9,16-20] but mostly by promoting endocytosis.[9] We reasoned that acylation with a *cis*-unsaturated fatty acid should promote direct translocation over endocytosis. Single or multiple *cis*-unsaturation(s) must decrease the aggregation propensity, (which was shown on cells to induce endocytosis),[9] and should participate to the "interfacial activity" of the peptide,[21] favouring its insertion within disordered domains and meanwhile locally perturb PL packing (increasing direct translocation).[15]

The scope of this study was to compare the impact of acylation with *cis*-unsaturated *vs.* saturated fatty acid chain of a minimalist cationic peptide containing only four arginines and an acyl chain.[9] A *C*-terminal lysine was added to this short cationic sequence, for labelling with a small neutral fluorescent probe: 4-nitrobenzo-2-oxa-1,3-diazole (NBD).[11-13] We showed by FACS and confocal microscopy that the acylation of $(Arg)_4$-Lys(NBD)-NH_2,with an all-*cis*-C22:6 fatty acid dramatically enhanced the internalisation at 4°C in four different cell lines, compared to C18:0, C18:1, C18:2, C12:0 and C12:1 acyl chains. The cellular uptake by direct translocation was selectively increased for the C22:6 lipopeptide, as ascertained by diffuse cytosolic and dense Golgi localisations at both 4°C and 37°C. (Footnote §).



Scheme 1. Formula and abbreviations of the NBD-labelled lipopeptides.

The length of the fatty acid chain and the number of unsaturations were varied from C12 to C22, and from 1 to 6, respectively. The commercially available C22:6 fatty acid (docosahexaneoic acid) was chosen, this ω3-fatty acid represents about 40% of the polyunsaturated fatty acids in the brain,[22] and it should be the best compromise between the length and the number of unsaturations to decrease the aggregation by micellisation of long fatty acyl chain.

[a] Sorbonne Universités, UPMC Univ Paris 06, Laboratoire des Biomolécules, UMR 7203, 4, Place Jussieu 75005 Paris, France
[b] Ecole Normale Supérieure – PSL Research University Institution, Département de Chimie, 24, Rue Lhomond, 75005 Paris, France
[c] CNRS – UMR7203, Paris, France
[d] INSERM - ERL 1157, Paris, France
[e] AP-HP, Hôpital Saint Antoine, 75012 Paris, France
[f] INRA, UR892, Virologie et Immunologie Moléculaires, 78350, Jouy-en-Jossas, France.
† J.-M. Swiecicki and M. Di Pisa: equal contribution
* Corresponding authors: solange.lavielle@upmc.fr, fabienne.burlina@upmc.fr

Electronic Supplementary Information (ESI) available: All the experimental procedures are described in SI with the characterization of all the lipopeptides. See DOI: 10.1039/x0xx00000x

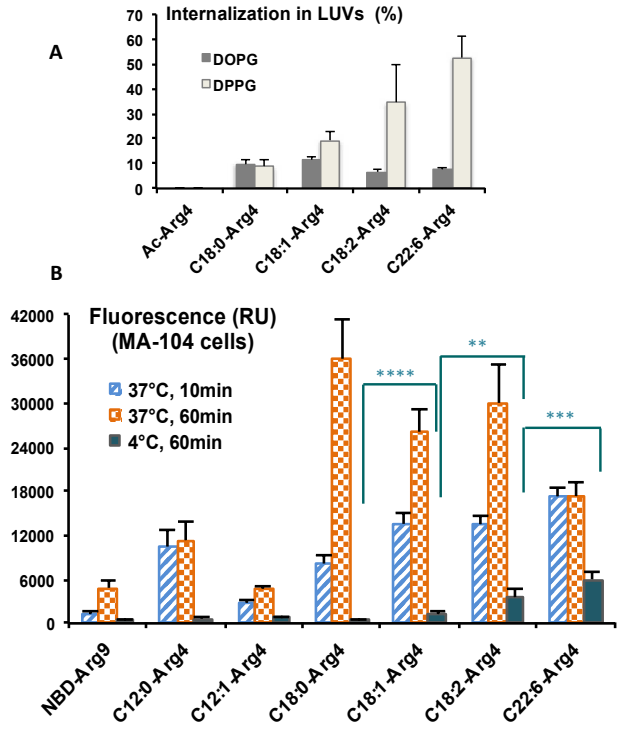


Fig. 1. A) Internalisation of the indicated NBD-labelled peptides (100 nM) into LUVs (100 nm, 10 μM, peptide:phospholipid ratio 1:100) made of 100% DOPG (at 20°C, fluid phase) or 100% DPPG (at 37°C, fluid domains in a gel phase), data are means of 3-5 experiments ± s.d., (*Cf.* S4 in SI).[11] B) Uptake into MA-104 cells (10^5 cells) at 4°C or 37°C of the indicated NBD-labelled peptides (5 μM) analysed by FACS. At the end of the incubation (10 or 60 min), dithionite was added to quench the fluorescence of the non-internalized NBD-peptides. For untreated cells and cells incubated with the control peptide Ac-Arg_4, data are reported in the SI, (*Cf.* S7 in SI). Data are means of 2-4 experiments ± s.d. Student test: with **: $p \leq 0.01$, ***: $p \leq 0.001$ and ****: $p \leq 0.0001$, respectively.

All the lipopeptides, C12:0-Arg4, C12:1-Arg_4, C18:0-Arg_4, C18:1-Arg_4, C18:2-Arg_4 and C22:6-Arg_4, (Scheme 1), were prepared *via* Boc strategy on solid support. The NBD, on the εNH_2 of the lysine, and the saturated acyl chains C12:0 and C18:0 at the *N*-terminus were introduced on solid support. The *N*-hydroxysuccinimic ester of the unsaturated acyl chains, C12:1, C18:1, C18:2 and C22:6, had to be coupled in solution to the crude precursor $(Arg)_4$-Lys(NBD)-NH_2. The final peptides and lipopeptides were purified either by HPLC or by Fast Centrifugal Partition Chromatography, (*Cf.* Footnote ‡ and S1 in SI). Two control peptides were also used in this study: Ac-$(Arg)_4$-Lys(NBD)-NH_2, abbreviated as Ac-Arg_4 (with Ac: acetyl) and the *N*-ter modified nona-arginine: NBD-$(Arg)_9$-NH_2, already used in previous experiments with LUVs.[11]

The internalisation of the lipopeptides was first analysed with two model systems, which have been found to be the most appropriate ones to screen the entry of CPPs.[14] LUVs (100 nm) were prepared from either 100% 1,2-dioleyl-*sn*-glycero-3-phosphoglycerol, (DOPG) or 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol, (DPPG).

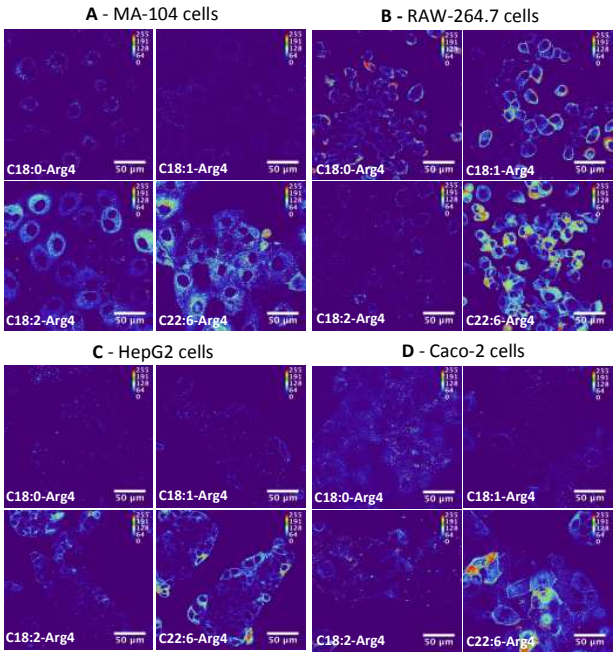


Fig. 2. Confocal laser scanning microscopy experiments. Internalisation of lipopeptides C18:0-Arg_4, C18:1-Arg_4, C18:2-Arg_4 or C22:6-Arg_4, with four cells lines (10^5 cells *per* dish): A) MA-104 cells, B) RAW 264.7 cells, C) HepG2 cells and D) Caco-2 cells. Peptides (5 μM) were incubated at 4°C for 60 min, the non-internalised NBD-labelled peptide was quenched by dithionite. Direct observation after washings.

After 5 min incubation of the LUVs (10 μM) with the fluorescent peptide (100 nM, peptide:phospholipid, ratio 1:100), the non-permeant reducing anion dithionite (DT) was added to quantitatively reduce and thus quench the fluorescence of the NBD-labelled peptide, which has not been internalised inside LUVs,[11] (Fig. 1A and *Cf.* S4 in SI). The lipopeptides accumulate to a similar extent inside LUVs in fluid phase (DOPG, 20°C). Interestingly, the accumulation of the lipopeptides in LUVs presenting fluid domains in gel phase (DPPG, 37°C) increases with the number of unsaturations; from C18:0-Arg4 to C18:2-Arg4 and C22:6-Arg4. Altogether the validity of our hypothesis was ascertained: unsaturations favour translocation of a short cationic lipopeptide into LUVs presenting nanodomains with packing defects, such as LUVs made of DPPG incubated at 37°C.

Our hypothesis was then confronted to the complexity of living cells, using four different cell lines: MA-104 cells (monkey kidney epithelial cells), RAW 264.7 cells (mouse leukemic monocyte macrophage), HepG2 cells (human hepatocellular carcinoma) and Caco-2 cells (epithelial cells from a human colorectal adenocarcinoma) and four lipopeptides C18:0-Arg_4, C18:1-Arg_4, C18:2-Arg_4 and C22:6-Arg_4.

These NBD-labelled peptides at concentrations from 1 to 10 μM did not affect the viability of the cells, only a slight but significant decrease in cell viability was observed at 10 μM for C18:0-Arg_4 and C18:1-Arg_4, on RAW 264.7 and HepG2 cells, (*Cf.* S6 in SI).

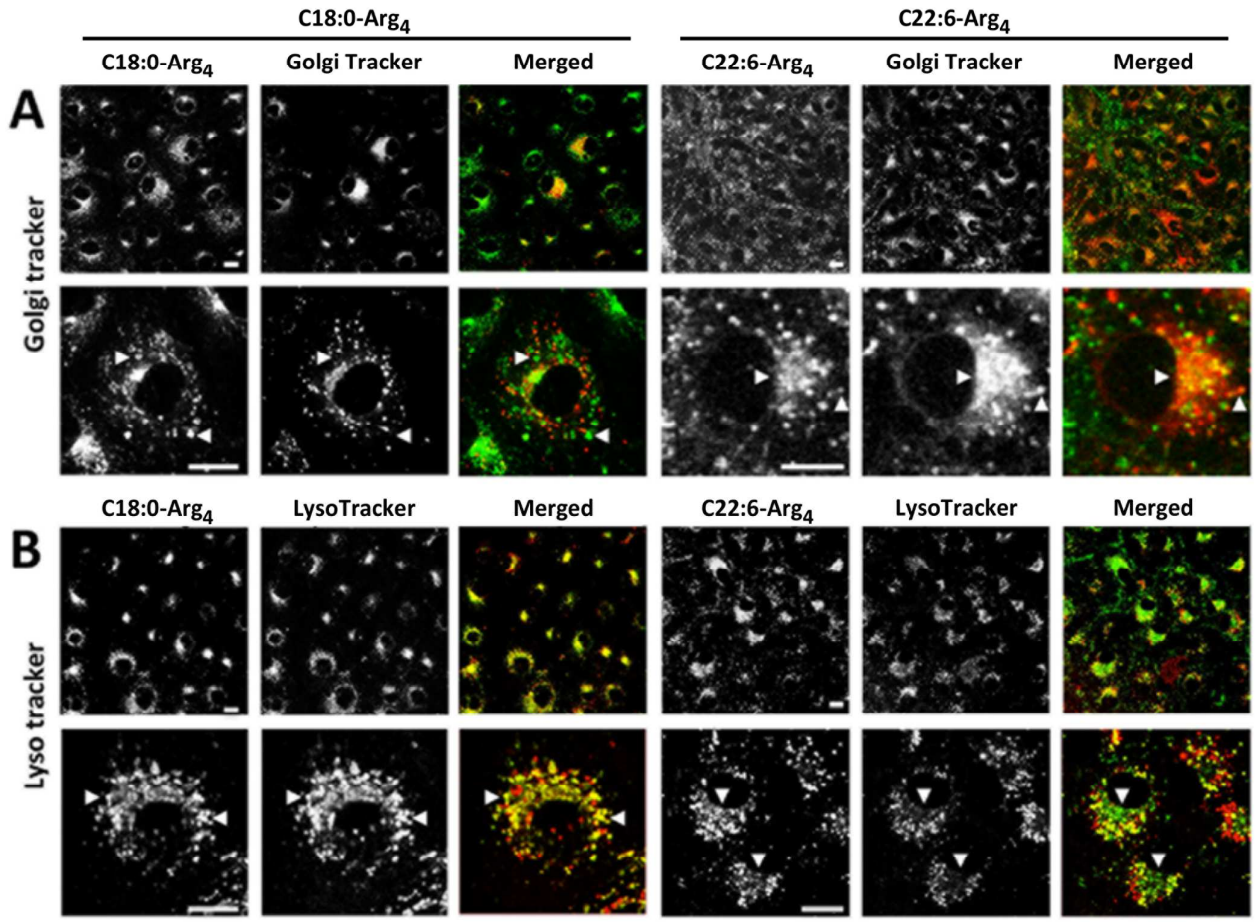


Fig. 3. Colocalisation studies of NBD-labelled C18:0-Arg_4 or C22:6-Arg_4 with either Golgi Tracker or Lyso Tracker using confocal laser scanning microscopy. After incubation with Golgi Tracker (A) or Lyso Tracker (B) followed by incubation with the indicated lipopeptide, cells were immediately treated with dithionite (3 min) to quench the staining of labelled peptides remaining at the cell surface. Upper panels of A and B correspond to the medial section of an image stack of a field observed with an x63 objective, while lower panels of A and B correspond to acquisitions performed at the same depth using a numerical zoom adjusted to observe a single cell, with for the merged pictures the lipopeptide in green (C18:0-Arg_4 and C22:6-Arg_4) and the tracker in red. A) MA-104 cells (10^5 cells), were incubated for 30 min at 4°C, then 30 min at 37°C with Golgi Tracker (Bodipy TR Ceramide, 5 μM), C18:0-Arg_4 (left panels) or C22:6-Arg_4 (right panels) were then added, at 5 μM, and further incubated for 60 min at 37°C. B) MA-104 cells (10^5 cells), were incubated for 30 min at 37°C with Lyso Tracker Deep Red (100 nM), C18:0-Arg_4 (left panels) or C22:6-Arg_4 (right panels) were added, at 5 μM, and further incubated for 60 min at 37°C.

Consequently, the lipopeptides were incubated at 5 μM, considering also the aggregation propensity of such amphiphiles, around 1 to 10 μM depending of the acyl chain, (*Cf.* S2 in SI). This concentration is also the best compromise for FACS and confocal microscopy experiments and the sensitivity of the NBD probe. In the literature, most of the experiments with CPPs, bearing different types of probe, are in the 1-20 μM range.[23]

The plasma membranes of the MA-104 cells incubated with lipopeptides (5 μM) are densely labelled, but this fluorescence was totally quenched by reduction/quenching of the NBD fluorophore with dithionite, to only detect the internalised fluorescent peptides. This quenching of fluorescence on the external leaflet was quantitative, as shown by flow cytometry and confocal microscopy assays (*Cf* S7 and S8 in SI). The internalisation into MA-104 cells was then analysed by flow cytometry after incubation for 10 or 60 min at 37°C, and 3 min reduction with dithionite (Figure 1B). Since Ac-Arg4 is not internalized (*Cf.* S7 in SI), the acyl chain is required for the internalisation of this short cationic peptide as observed before.[9] Importantly, depending on the chain length and number of unsaturation(s), two trends are observed. C12:0-Arg_4 and C22:6-Arg_4 are rapidly internalised, with no increase in intracellular fluorescence after 10 min incubation, whereas for C18:0-Arg_4 a 4.8-fold increase in the intracellular fluorescence is observed when increasing the incubation time from 10 to 60 min. The delayed internalisation for C18:0-Arg_4, as also observed for NBD-Arg_9, should be related to a slow onset of the endosomal machinery. These data therefore suggested that the main internalisation pathway differs for both groups of CPPs. This was confirmed by incubating the lipopeptides at 4°C.

Indeed, at 4°C, the internalisation *via* active mechanisms (endocytosis) is abolished, and incubation at this temperature allowed to unveil the involvement of direct translocation, an energy independent mechanism. The fluidity of the plasma membrane should also change at 4°C, but translocation can still occur even if probably reduced. Interestingly, at 4°C the amount of internalisation *via* direct translocation increases significantly with the number of insaturation(s) from C18:0-Arg_4 to C18:1-Arg_4 and C18:2-Arg_4. The most efficient lipopeptide at 4°C was found to be as hypothetised C22:6-Arg_4, when the C18:0-Arg_4 lipopeptide and the control NBD-Arg_9 are poorly internalised. Thus, the direct translocation pathway is dramatically enhanced for the polyunsaturated C22:6-Arg4. The entry at 4°C of the four lipopeptides C18:0-Arg4, C18:1-Arg4, C18:2-Arg4 and C22:6-Arg4 was further analysed with the four different cell lines by confocal microscopy (Fig 2). In every cases the most unsaturated lipopeptide C22:6-Arg4 was found to be the best one in the four cell lines with an intense labelling of the cells at 4°C after 60 min incubation, even though C18:2-Arg4 was not always better than C18:1-Arg4 (this difference was systematically observed with RAW cells). Furthermore, most of the cells are labelled with the C22:6-Arg4, showing that the internalisation was homogenous at that concentration and peptide/cell ratio.

The intracellular localisations of C18:0-Arg_4 and C22:6-Arg_4 were compared by confocal microscopy after 60 min incubation with MA-104 cells at 4°C or 37°C. At 4°C, the internalisation of C18:0-Arg_4 is too faint to allow co-localisation experiments, (Fig. 2A). In contrast, at 4°C the cytosol of the MA-104 cells is densely labelled by C22:6-Arg_4, (Fig. 2A) and most of the fluorescence co-localised with the Golgi membranes, but not at all with lysosomes (*Cf.* S8 in SI). At 37°C, C18:0-Arg_4 is internalised to a larger extent in MA-104 cells, however most of the fluorescence is punctuated and co-localised with lysosomes, (Fig. 3). In contrast, the fluorescence of C22:6-Arg_4 in MA-104 cells is more diffuse in the cytosol and with a dense labelling, which co-localised with the Golgi membranes (Fig. 3A), but barely with lysosomes (Fig. 3B). Altogether, these data showing a faster entry by direct translocation and a cytosolic distribution for C22:6-Arg_4 validate the original hypothesis: unsaturated chain promotes for this short oligo-arginine lipopeptide membrane translocation *vs.* endocytosis. It might be hypothesized that if the lipopeptide were internalised by endocytosis the presence of unsaturations might for the same reasons promote its endosomal escape.

Approaches to improve intracellular targeting involve either a direct transfer through the plasma membrane or/and a necessary escape from endosomes. Acylation of a short cationic peptide by *cis* unsaturated chain(s) provides at last a new rational CPP modification,[24] to promote cytosolic localisation by favouring the direct translocation pathway.

Notes and references

§ This work was supported in part by funding from ANR: J12R139 "ELIPTIC". The authors acknowledge Dr. A.-M. Faussat, R. Morrichon and A. Munier from the Plate-Forme d'Imagerie Cellulaire Microscopie Confocale, UMPC - IFR 65.

‡ Syntheses of the lipopeptides: Boc-Arg(Tos)$_4$-Lys(Fmoc)-MBHA was synthesised by SPPS on a MBHA Resin. Activation was accomplished with DCC/HOBt in NMP. After Fmoc deprotection by piperidine, the fluorophore was introduced on the lysine side chain by reacting, in DMF, NBD-Cl (4 eq.) with DIEA (10 eq.). The saturated acyl chains were introduced at the *N*-terminus of this peptidyl resin, after Boc deprotection by trifluoroacetic acid, and on-resin acylation of the ⍰⍰⍰⍰⍰ arginine with the corresponding fatty acid pre-activated by DCC/HOBT. The final lipopetides were cleaved from the support by HF in the presence of anisole and Et_2S. For the preparation of the unsaturated lipopeptides, Arg_4-Lys(NBD)-NH_2 was obtained after cleavage from the support and this crude peptide was acylated in solution with the corresponding *N*-hydroxysuccinimic ester of the unsaturated fatty acid. The crude peptides were lyophilised before purification by HPLC or Fast Centrifugal Partition Chromatography leading to purity ≥ 97%. The purified peptides were characterised by MALDI-TOF mass spectrometry, (*Cf.* S1 in SI).

1 A. Prochiantz, *Trends Cell Biol.,* 1998, **8**, 84.
2 S. Pujals, J. Fernandez-Carneado, C. Lopez-Iglesias, M. J. Kogan, E. Giralt, *Biochim. Biophys. Acta,* 2006, **1758**, 264.
3 F. Madani, S. Lindberg, Ü. Langel, S. Futaki, A. Gräslund, *J. of Biophysics,* 2011, Article ID 414729.
4 E. Koren, V. P. Torchilin, *Trends Mol. Med.,* 2012, **18**, 385.
5 A. T. Jones, E. J. Sayers, *J Control Release*, 2012, **161**, 582.
6 I. Nakase, H. Akita, K. Kogure, A. Graslund, U. Langel, H. Harashima, S. Futaki, *Acc. Chem. Res.,* 2012, **45**, 1132.
7 E. G. Stanzl, B. M. Trantow, J. R. Vargas, P. A. Wender, *Acc. Chem. Res.,* 2013, **46**, 2944.
8 C.-Y. Jiao, D. Delaroche, F. Burlina, I. D. Alves, G. Chassaing, S. Sagan, *J. Biol. Chem.,* 2009, **284**, 33957.
9 S. A. Bode, M. Thévenin, C. Bechara, S. Sagan, S. Bregant, S. Lavielle, G. Chassaing, F. Burlina, *Chem. Commun.,* 2012, **48**, 7179.
10 J.-P. Berlose, O. Convert, D. Derossi, A. Brunissen, G. Chassaing, *Eur. J. Biochem.,* 1996, **242**, 372.
11 J.-M. Swiecicki, A. Bartsch, J. Tailhades, M. Di Pisa, B. Heller, G. Chassaing, C. Mansuy, F. Burlina, S. Lavielle, *ChemBioChem,* 2014, **15**, 884.
12 M. Di Pisa, G. Chassaing, J.-M. Swiecicki, *Biochemistry,* 2015, **54**, 194.
13 M. Di Pisa, G. Chassaing, J.-M. Swiecicki, *J. Pept. Sci.*, 2015, **21**, 356.
14 J.-M. Swiecicki, M. Di Pisa, F. Burlina, P. Lécorché, C. Mansuy, G. Chassaing, S. Lavielle, *Biopolymers*, 2015, DOI 10.1002/bip.22652.
15 V. T. Armstrong, M.R. Brzustowicz, S. R. Wassall, L. J. Jenski, W. Stillwell, *Arch. Biochem. Biophys.,* 2003, **414**, 74.
16 S. Futaki, W. Ohashi, T. Suzuki, M. Niwa, S. Tanaka, K. Ueda, H. Harashima, Y. Sugiura, *Bioconjugate Chem.,* 2001, **12**, 1005.
17 W. Pham, M. F. Kircher, R. Weissleder C. H. Tung, *ChemBioChem,* 2004, **5**, 1148–1151.
18 J. S. Lee, C. H. Tung, *Mol. Biosyst*., 2010, **6**, 2049.
19 S. Katayama, H. Hirose, K. Takayama, I. Nakase S. Futaki, *J. Control Release,* 2011, **149**, 29.
20 D. Oh, A. N. Shirazi, K. Northup, B. Sullivan, R. Tiwari, K. M. Bisoffi, K. Parang, *Mol. Pharmaceutics,* 2014, **11**, 2845.
21 W. C. Wimley, *ACS Chem. Biol*., 2010, **5**, 905.
22 J.T. Green, S. K. Orr, and R. P. Bazinet, J. Lipid Res., 2008, **49**, 939.
23 F. Duchardt, M. Fotin-Mleczek, H. Schwarz, R. Fischer, R. Brock, *Traffic,* 2007, **8**, 848.
24 D. M. Copolovici, K. Langel, E. Eriste, Ü. Langel, *ACS Nano*, 2014, **8**, 1972.