Arginine-rich cell-penetrating peptides induce membrane multilamellarity and subsequently enter via formation of a fusion pore

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Arginine-rich cell penetrating peptides do not enter cells by directly passing through a lipid membrane, they instead passively enter vesicles and live cells by inducing membrane multilamellarity and fusion. The molecular picture of this penetration mode, which differs qualitatively from the previously proposed direct mechanism, is provided by molecular dynamics simulations. The kinetics of vesicle agglomeration and fusion by an iconic cell penetrating peptide - nonaarginine - is documented via real time fluorescence techniques, while the induction of multilamellar phases in vesicles and live cells is demonstrated by a combination of electron and fluorescence microscopies. This concert of experiments and simulations reveals that the newly identified passive cell penetration mechanism bears analogy to vesicle fusion induced by calcium ions, indicating that the two processes may share a common mechanistic origin.

Cell penetrating peptides | Vesicle fusion | Ion membrane interactions | Molecular dynamics | Fluorescence microscopy | Electron microscopy

Cell penetrating peptides have a unique potential for targeted drug delivery, therefore, mechanistic understanding of their membrane action has been sought since their discovery over 20 years ago.(1) While ATP-driven endocytosis is known to play a major role in their internalization(2) , there has been also ample evidence for the importance of passive translocation(3–5) for which the direct mechanism, where the peptide is thought to directly pass through the membrane via a temporary pore, has been widely advocated. (4, 6–8) Here, we question this view and show that arginine-rich cell penetrating peptides instead passively enter vesicles and live cells by inducing membrane multilamellarity and fusion.

Ions do not dissolve in oil. From this point of view the direct passive mechanism of cell penetration is intuitively problematic, as cationic peptides such as polyarginines or the trans-activating transcriptional activator (TAT) are too highly charged to be able to pass through the "oily" interior of a lipid membrane. The concept of direct penetration was seen plausible due to the action of the related antimicrobial peptides, which are also charged, but in addition contain a large fraction of hydrophobic residues(9): These peptides are known to stabilize pores in membranes(10). At a close inspection, however, it becomes clear that their charged sidechains do not interact directly with the aliphatic chains in the low dielectric interior of the phospholipid bilayer, but rather stabilize transient water channels or act as terminal residues anchoring the transmembrane helix.(9) Taken together, the passive action of cell penetrating peptides (CPP) seems to be very different from direct translocation across an otherwise unperturbed cell membrane.

To make matters even more confusing, experimental facts and suggested mechanisms often seem contradictory to each other. For example, there are conflicting reports whether or not nonaarginine (R_9) is able to penetrate vesicles composed purely of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC). (5, 11, 12) Additionally, fluorescence microscopy suggests that R_9 is able to deform membranes(5, 13) and small angle X-ray scattering (SAXS) experiments reveal phase transitions induced in lipid systems by polyarginines.(4) An important factor in these observations appears to be the membrane composition with negatively charged lipids facilitating membrane translocation of cationic peptides.(7, 14) Indeed, there is some evidence that a direct mechanism may be enabled by hydrophobic counterions, such as pyrene butyrate(12, 15) or presence of an unphysiological concentration of phosphatidic acids.(7) The relevance to of these phenomena to actual cellular uptake is not clear, so that current discussions present direct mechanisms

Significance Statement

The passive translocation mechanism of arginine-rich cell penetrating peptides has puzzled the scientific community for more than twenty years. In this study we propose a new mechanism of passive cell entry involving fusion of multilamellar structures generated by the cell penetrating peptides. The geometry of entry for this mechanism is completely different from previously suggested direct translocation mechanisms leading to a new paradigm for designing molecular carriers for drug delivery to the cell.

A.M., C.A. and M.J. performed the simulations, supervised by P.Jun and D. H., vesicle fluorescence related experiments and DLS were performed by P.Jur., C.A., K.B. and supervised by M. H. fluorescence data were interpreted by C.A. and R.S. Cryoelectron microscopy on LUVs was performed by V.H. and R.R., supervised by C.Z.; fluorescence and electron microscopy on HeLa cells was done by M.C., A.S. and P.E.M.; simulation visualizations were done by A.M. and C.A.; C.A. and P.Jun wrote the paper.

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side by side with endocytosis-like membrane deformations induced by the CPP.(16)

Another fundamental cellular process involving membranes and charged species is fusion of vesicles with the cell membrane during calcium-triggered exocytosis. In cells, vesicle-membrane fusion is mediated by the SNARE protein complex(17, 18) with synaptotagmins(19), nevertheless, it can also be induced in *in vitro* lipid vesicles without the need for the presence of the protein machinery(20, 21). It is experimentally well established that Ca^{2+} is a key player capable of promoting vesicle fusion(22) and there is general consensus about the fusion mechanism, which proceeds via a stalk intermediate, followed by formation of a hemifused structure and opening of a fusion pore.(23, 24) In this context, it is worth mentioning that cationic cell penetrating peptides, especially TAT and its derivatives, are known to aggregate at phospholipid membranes and occasionally fuse vesicles.(2, 5, 20, 25) This brings up the idea, which is examined further in this study, that the processes of passive cell penetration and membrane fusion may be mechanistically more intimately connected than thought so far.(25)

Results and Discussion

Exploring vesicle penetration by a fluorescence leakage essay. In order to explore the potential connection between cell penetration and membrane fusion, we start by investigating the abilities of R_9 as an archetypal CPP, in contrast to non-CPPs like tetraarginine (R_4) or nonalysine(26) (K_9), to penetrate and cause leakage of large unilamellar vesicles (LUVs) of varying lipid compositions using a fluorescence leakage assay (for details see Methods and the SI Appendix). While, contrary to some published data(12), pure POPC LUVs do not show leakage upon exposure to R_9 even at high peptide concentrations, LUVs composed of mixtures of 1,2-dioleoyl-phosphatidylethanolamine (DOPE) and 1,2-dioleoyl-phosphatidylserine (DOPS) exhibit leakage as long as the content of DOPE is sufficiently high (see Table S3 in the SI Appendix). Despite different lipid composition of the present vesicles compared to live cells, we did recover the arginine 'magic', i.e., the high activity of CPPs with more than about seven aminoacids and high arginine content (27). In these vesicles, similarly to experiments in cells, R_9 was always found to be an efficient leakage agent, in contrast to K_9 or R_4 (see Figure 1, top left). Using dynamic light scattering (DLS) measurements we showed that leakage is accompanied with vesicle aggregation, as the leakage kinetics mimics the increase in time of the mean diameter of the aggregates (see Figure 1, top right and the SI Appendix, Table S3 for the DOPE-rich lipid composition).

Membrane fusion induced by calcium as well as by cationic cell penetrating peptides. The range of lipid compositions of vesicles capable of being leaked by R_9 is at odds with simulations of direct translocation, where a far higher translocation free energy has been predicted for DOPE-rich bilayers than for those rich in POPC.(28) However, it seems to match compositions known to enhance vesicle fusion by calcium.(20, 21, 29) Both PE and PS (as well as several other anionic lipids) are fusogenic in presence of Ca^{2+}.(30–32) In order to verify this correlation, we repeated the experiments with Ca^{2+} instead of R_9. Indeed, we were able to observe calcium-induced leakage for lipid compositions that were most susceptible to leakage by the CPPs as well (see Table S3 in the SI Appendix). In order to obtain a comparable effect, however, the concentration of Ca^{2+} had to be significantly higher than that of R_9, even when taking into account the significantly larger charge carried by the latter species.

At high peptide content, the LUV leakage kinetics is described *quantitatively* by a second order rate law in the vesicle concentration (for details see Figure S11 and the kinetic model in the SI Appendix). This indicates that aggregation of vesicles and the double bilayer formed during this process is essential for vesicle leakage. It is indicative that for all lipid compositions at which significant leakage occurs the vesicles also exhibit R_9-induced fusion, as detected by a near complete lipid mixing in a Förster resonance energy transfer (FRET) assay (see the SI Appendix, esp. Figure S11). Moreover, the leaky lipid compositions are those known to be susceptible to Ca^{2+} mediated fusion. Note that leaky fusion has been observed previously in conjunction with cell penetrating peptides and has even been used to classify them.(25) In addition to the above circumstantial evidence for a direct connection between cell penetration and membrane fusion we add further experimental support using confocal microscopy finding agglomeration of giant unilamellar vesicles (GUVs) together with leakage (see Figure 1). We were able to observe fusion of GUVs directly (see the Supporting Video) and, using Oregon Green 488 (OG)-labeled peptides, we confirmed a preferential adsorption of fluorescently labeled R_9 to the vesicles (see Figure S8 in the SI Appendix). Finally, adding Ca^{2+} instead of R_9 to the GUVs we found a functionally analogous behavior (see the SI Appendix).

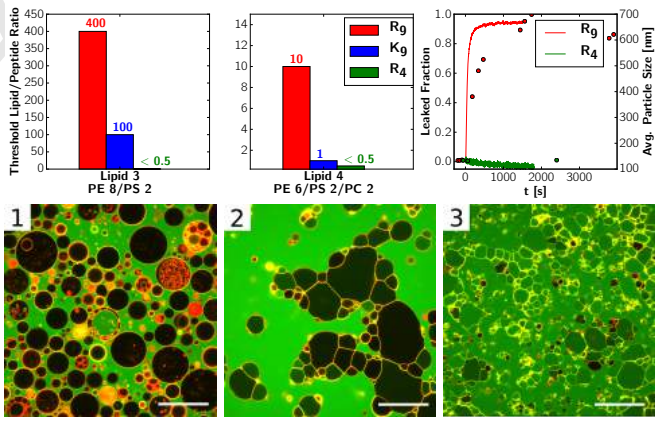

Fig. 1. Fluorescence spectroscopy results. Top Left: Threshold concentrations for leakage induced by R_9, K_9 and R_4 given as inverse of the peptide/lipid ratios for two lipid compositions: DOPE/DOPS 80/20 (3) and DOPE/DOPC/DOPS 60/20/20 (4) (the higher the threshold value, the more efficient the peptide is in leaking the vesicles). Top Right: DLS measurements showing particle growth (right axis, dots) overlayed with leakage kinetics (left axis, lines) for R_9 for the composition (3) and absence of particle growth and leakage for R_4. Bottom panel: Fluorescence microscopy images showing the effect of R_9 on GUV with composition (4). From left to right: **1**. No peptide added, **2**. Shortly after addition of R_9 **3**. Final state after 1h. Bars = 50 μm.

Ideal fusion topologically precludes cell penetration. The similarities in aggregation/fusion caused by R_9 and Ca^{2+} are illustrated in Figure 2, **A**-**J**. In the context of the present study, it is important to note that there is no topological way for peptides to enter the vesicles from the outside (or do the reverse) by an ideal fusion process, within which two unilamellar vesicular structures coalesce, as it merely connects the

interiors of the two vesicles. A previous electron microscopy study of Ca^{2+}-mediated fusion reported content loss along the fusion diaphragm, attributing it to the strain induced by the deformation during agglomeration.(33). A close observation of the GUVs in Figure 1 indeed reveals that they are significantly deformed as the agglomeration creates nearly planar surfaces at the regions of contact, indicating high tension. While tension supports several fusion steps (34), the large surface tension of the agglomerated, flat, and fusing double bilayers can lead to rupture - depending on the initial tension of the vesicles it may overcome the line tension leading to a membrane pore. However, a small, transient, pore near the fusion stalk can hardly be the main mechanism of penetration of peptides into vesicles since it will rapidly close (35, 36), making it hard for cargo to pass through. Such pores are also associated with positive curvature, as induced by certain amphipathic helices(37), whereas we stabilize fusion stalk and pore with PE lipids (23) inducing negative curvature and the hydrophilic R_9. For a cell membrane to be ruptured a large enough membrane fold for self-fusion would have to be generated first. This is only possible for a system without a large surface tension. However, such membranes are unlikely to be ruptured by a fusion event. Therefore, rupture cannot explain continuous leakage into intact vesicles, nor is it a plausible mechanism for cell penetration as cells tightly regulate their inner pressure and membrane tension and are rich in cholesterol, which increases pore line tension. We show below that a solution lies in locally bifurcating the membrane, leading to multilamellar structures. Such a pathway allows the peptides to enter by fusion without having to form transient pores, as shown schematically in Figure 2, **K**-**V**.

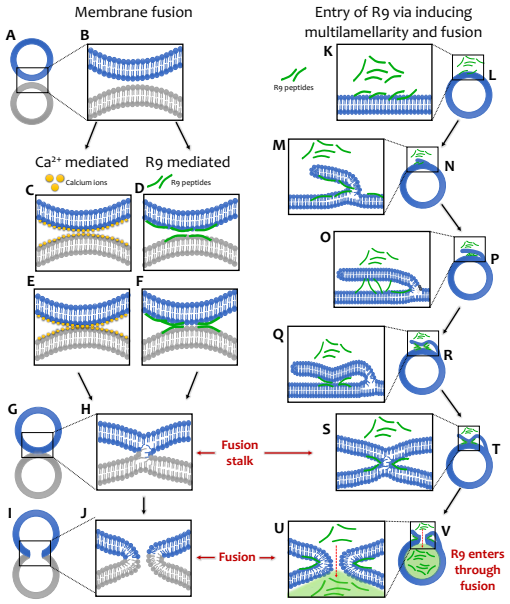

Fig. 2. The schematic mechanisms of R_9 and Ca^{2+} mediated vesicle fusion: **A** Fusion of different vesicles (in blue and grey), by **B** interface contact. **C, D** Adsorption of the charged particles (R_9 in green and Ca^{2+} in yellow). **E, F** Agglomeration of the bilayers induced by crosslinking. **G** Stalk formation. **H** Opening of the fusion pore. **I, J** R_9 translocation via self-fusion of a single vesicle, **K-V** starting from a flat vesicle surface bilayer. **K** Strong adsorption of R_9. **K, L** Membrane bifurcation through adhesion and curvature.**M, N** Extension of the bifurcated bilayer through R_9 crosslinking **O, P**. Agglomeration of the bilayers induced by crosslinking of two bilayers on the same vesicle **Q, R**. Stalk formation **S, T** and opening of the fusion pore through which additional R_9 peptides enter **U, V**.

Induced multilamellarity as a solution to the topological conundrum.

Cryo-electron microscopy: Seeing is believing. A tendency of GUVs(13) or cells(12) to become multilamellar upon addition of CPPs has been observed recently. To further explore this idea, we first conducted cryo-EM experiments on LUVs. The obtained cryo-EM images indeed reveal formation of multilamellar domains and lipid bilayer bifurcations after the addition of R_9, see Figure 3. Additional time-resolved FRET experiments on fluorescently labeled LUVs reveal presence of inter-bilayer energy transfer, which provides independent confirmation for the induction of multilamellar lipid structures by R_9 (see the SI Appendix and Figure S11 therein). Importantly, Ca^{2+} ions are also able to fuse and collapse vesicles to multilamellar phases due to the ability of Ca^{2+} to bridge phosphates from different bilayers.(33) The cryo-EM structures also provide some additional evidence for fusion, as the LUVs in Figure 3, A are many times larger than those found in the initial state (again see Figure S7, A).

Multilamellar structures can be formed via folding of a membrane or by stacking of deflated vesicles. Any process based on direct membrane stacking would, however, add an even number of bilayers in between the vesicles and, therefore, would not lead to leakage via fusion. It is thus a key finding that by counting the lipid bilayers we frequently find odd numbers (see Figure 3, D). Moreover, a close inspection of the EM micrographs provides direct evidence for bilayer bifurcation at multiple positions (see Figure 3, C for an example). We conclude that R_9 is indeed capable of inducing multilamellarity by membrane adsorption and bifurcation, rendering a cell penetration mechanism via fusion feasible.

The proposed mechanism shares some similarities with the reverse micelle mechanism, proposed in the literature(38, 39). This mechanism also necessitates a small bifurcation, before the membrane edge is closed by forming the reverse micelle. The reverse micelle has negative curvature on the inside and is, therefore, stabilized by similar interactions as the bifurcations. We argue that the membrane edge energy can be compensated through extension of stable cross-linked multilamellar domains as seen in the EM pictures. In the SI we show simulations, which indicate the stabilization of the bifurcation by R_9, but not R_4 even in the absence of crosslinking. The opening of a reverse micelle removes negative curvature from the system. In contrast to this, the fusion stalk(23) and pore both maintain a negative curvature – a finite bilayer thickness translates negative Gaussian curvature into negative mean curvature, present on both membrane leaflets.(40) Thereby the whole mechanism can be driven by the same preferential interaction with R_9.

Fluorescence and electron microscopy on HeLa cells. In order to directly explore the mechanism behind cellular uptake of CPPs in the absence of endocytosis, we first observed penetratation into living human HeLa cells by fluorescence confocal microscopy (Figures S9, S10 in the SI Appendix). HeLa cells incubated with 15 μM Oregon-Green (OG) labeled-R_9 peptide for 3 minutes at 4 °C already exhibited surface fluorescence and, in particular, the presence of highly fluorescent foci (SI Appendix, Figure S9, arrows). Longer incubation of cells with the peptide increased the number of foci detected on HeLa cells. Cytosolic presence of the peptide was observed as soon

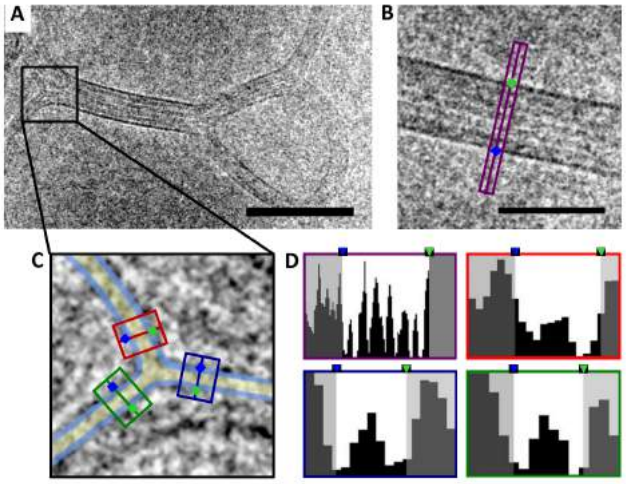

Fig. 3. Electron micrographs of LUVs in the presence of R_9. (**A**): vesicles treated with R_9 (>60 sec.) fuse with each other and exhibit bifurcated, multilamellar membranes; Bar = 100 nm. (**B**) Example of a multilamellar membrane. Bar = 50 nm.; The violet box is analyzed in (**D**). (**C**): Example of a membrane bifurcation. The membranes before and after the bifurcation site are analyzed by line-scans. The line-scan areas are marked with colored boxes. (**D**): The histograms are boxed in the same color as the respective line-scan areas in (**B**) and (**C**). The histogram (upper left panel) corresponding to the multilamellar membrane (shown in (**B**)) exhibits seven distinct minima attributed to individual membranes.

as 5 min after addition of the peptide to the cells and the number of penetrated cells increased over prolonged incubation time (even when cells were washed after 3 minutes to remove free peptide from incubation medium). All cells with cytosolic peptide exhibited at least 1-2 fluorescent foci on their surfaces. Moreover, these foci were found only in cells with exocytosis blocked by low temperature.

These results suggest that R_9 rapidly accumulates in a very few places on a cell at low temperatures. Previously, electron microscopy (EM) on cells revealed that addition of R_9 leads to the formation of layered membrane domains.(41) Motivated by these observations, we performed EM experiments on HeLa cells with added fluorescently labeled R_9. In accord with our observations on vesicles and with previous EM on cells(41) we see regions of protrusions, bifurcations, and multilamellarity by incubating cells with R_9 at 4 °C. At these conditions active endocytosis is switched off and only passive cell penetration is operational (see Figure 4, B). The branched structures (see zoomed-in images in Figure 4, C) are topologically identical to those suggested in the schematic drawing above (Figure 2, M) and to the structures observed in the LUVs (Figure 3). The observed protrusions indicate that a strong curvature-generating interaction is at work, as can be seen in Figure 4, D. Specifically, we interpret the budding spherical protrusions as being due to negative (Gaussian) curvature generation, similar as occuring in a fusion stalk. By overlaying fluorescence images with EM we clearly see that the changes in the bilayer structure correlate with the location of the fluorescently labeled OG-R_9, as can be seen in Figure 4, A. Our EM images on HeLa cells are thus consistent with the above results on vesicles, as well as with previous observations showing that giant plasma membrane vesicles (GPMVs) are susceptible to R_9 penetration, but only in the presence of intact membrane proteins.(3). Note that in contrast to our leakage experiments on LUVs the local, encapsulated foci will allow only very limited diffusion, thereby limiting potential cell toxicity of the CPP. Their locality at the cell membrane also points to a yet unknown specific interaction which is not present in the synthetic vesicle systems.

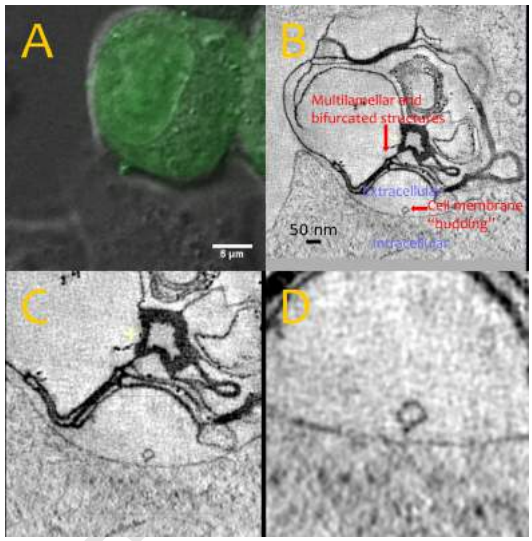

Fig. 4. Electron microcopic and fluorescence microscropy images of the same spot on a fixated HeLa cell in the presence of OG-R_9. (**A**): A fluorescence microscopy image of the multilamellar spot showing the presence of the labeled peptide. (**B-D**): An electron microscopy image at three zooms exhibiting bifurcated, multilamellar membranes and vesicle budding. (**C**) An example of a multilamellar membrane structure.; (**D**): Focus on a budding protrusion.

Molecular dynamics simulations: Atomistic insights. In order to gain atomistic insight into the fusion process and its connection to cell penetration we performed molecular dynamics simulations. Previous studies, based on continuum and coarse-grained models agree that fusion proceeds via a stalk intermediate. (23, 35) The stalk is strongly concave, explaining the observed lipid selectivity toward small (PE) headgroups as these stabilize negative curvature.

Our simulation setups involve strongly positively curved bilayer geometries, intended to lower the barriers for fusion.(42, 43) The stress hereby induced in the PE-rich bilayers leads to spontaneous stalk formation in our Simulations (see Methods and the SI Appendix for full details). Snapshots from the R_9 or Ca^{2+} mediated fusion processes are presented in Figure 5. We find both Ca^{2+} ions and the charged R_9 side chains to bind to lipid headgroups, primarily at the negatively charged phosphates. Subsequently, we observe mechanistic similarities in the membrane fusion mediated by Ca^{2+} and R_9. The first step in the fusion process is crosslinking, i.e., simultaneous binding of lipids from two membrane bilayers by either Ca^{2+} or R_9, with the latter being found to be a particularly effective crosslinker. As the ions keep the bilayers in close contact, the lipid tails eventually crosslink, too, in what appears to be the rate-determining step of the whole fusion process. This lipid tail crosslinking occurs within about half a microsecond. Once a cross-linking lipid tail has flip-flopped into the opposing bilayer, the stalk starts forming within a few nanoseconds (Figure 5, B and C).

In Figure 5, D we examine the action of R_9 on the membrane in close detail. First, we note the long-range crosslinking capability of R_9, which is likely to be responsible for its strongly agglomerating effect on vesicles and for stabilizing the multilamellar structures we find in electron microscopy. R_9 tends to be only partially adsorbed at the membrane and can

thus easily reach across the interface and attach to the second bilayer. It is clear that for such a crosslinking a minimum chain length is necessary, which explains the inefficiency of R_4 in this respect (see also the control simulation data, in Figure S3 in the SI Appendix). In addition, R_9 also forms agglomerates when crosslinking the interfaces. It is known that R_9 binding to membranes is more cooperative than that of K_9(44), making the former a much more efficient agent of membrane fusion and cell penetration than the latter. The attraction of R_9 to negatively charged lipids also leads to a lipid sorting effect, with phosphatidylserine lipids (Figure 5, D, orange) being accumulated next to the R_9 agglomerate. Electrostatics-based lipid sorting (involving, e.g., gangliosides) may contribute to the action in cells, membranes of which would otherwise not be sufficiently active. In the bottom right panel of Figure 5, we show that R_9 is preferentially adsorbed to regions of negative curvature(45) (marked by the color-coded surface), as this allows it to efficiently bind its sidechains to lipid headgroups (marked in gold). Aggregation of headgroups increases membrane tension in the headgroup region and exposes hydrophobic patches (visible in all panels of Figure 5, D). The exposure of hydrophobic patches in turn lowers hydration repulsion. Once lipid tails from opposing bilayers are in contact, lipid flip-flop can readily occur, starting thus the fusion cascade. At the surface of the fusion stalk and the pore, a very strongly negative Gaussian curvature and a negative mean curvature occur simultaneously. This stabilizes the actual geometry and, therefore, lowers the barrier for fusion. The induction of negative Gaussian curvature has previously been proposed as a characteristics of cell penetrating peptides.(4). Importantly for the present mechanism, we do not observe membrane rupture or water permeation during the fusion process.

Conclusions

In summary, we unraveled here a novel passive entry mechanism of cell penetrating peptides via branching and layering of membranes followed by fusion of the agglomerated systems. The layering is induced by a cooperative bridging of bilayers via adsorbed R_9. The peptides also induce membrane bifurcations that allow to connect the vesicle exterior and interior via fusion and thus to translocate the cell penetrating peptides (which would not be topologically possible within an ideal vesicle fusion). The actual R_9-induced fusion process then mirrors that of Ca^{2+} assisted vesicle fusion. The viability of this mechanism is supported by experimental results from electron microscopy, fluorescence microscopy, and light scattering, together with with molecular dynamics simulations. The atomistic simulation data shed further light on the molecular mechanism of formation of the fusion stalk and pore. While the new mechanism has been unraveled for lipid vesicle systems, which allow for investigations with unprecedented molecular detail, induction of membrane branching and multilamellarity by polyarginines has been observed by electron microscopy also in cellular membranes of live cells at low temperatures, indicating that the passive cell penetration process analogously involves membrane fusion.

Future work will be directed toward unraveling further molecular details of the cell penetration mechanism suggested in this discovery study. In the next step, we need to understand the interaction of cell penetrating peptides with biological cell surfaces. Increased experimental understanding of the specific binding will allow us to develop more realistic models and vice versa. This will not only allow to firmly establish all the details of this hithertho unrecognized mechanism of passive cell penetration, but will also have a direct impact on development of smart cell delivery strategies for therapeutic molecules employing cell penetrating peptides. Should we get this passive cell penetration mechanism under full control, we may eventually be able to expoit it to directly deliver cargo into the cell without the need for releasing it from the transport vesicles, as is the case in active endocytosis.

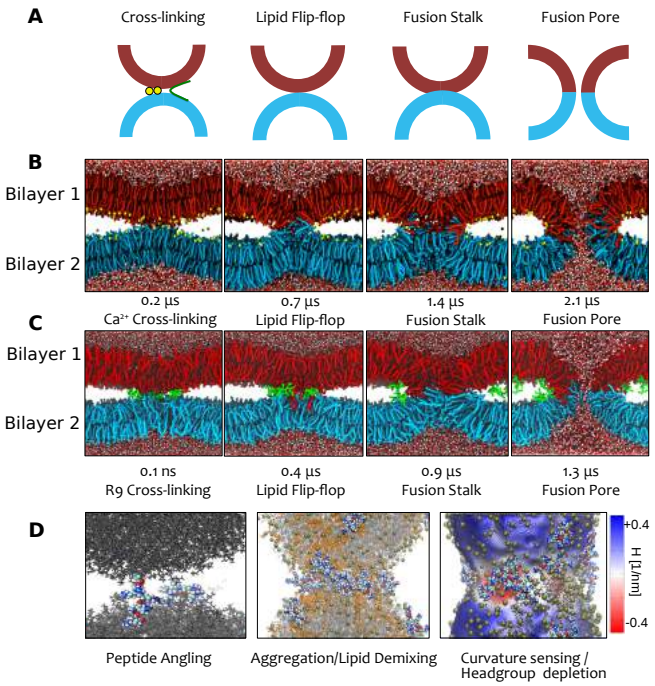

Fig. 5. A: Schematic drawing of vesicle fusion - lipid crosslinking, stalk initialization and subsequent onset of stalk formation through lipid flip-flop. **B**: Time evolution of the Ca^{2+} fusing bilayer system. **C**: Time evolution of the same system with R_9. Cross section of systems undergoing fusion: crosslinking, flip-flop, fusion stalk , fusion pore. **D**: Driving forces of the mechanism. Peptide "angling" crosslinks vesicles and aggregates membranes, peptide agglomeration and lipid demixing create fusable interface, generation of negative curvature through strong binding to headgroups.

Materials and Methods

Liposome Experiments. *Leakage*: Calcein containing vesicles were stirred at room temperature with LUV buffer in a quartz cuvette to obtain 1.5 ml of solution. The calcein fluorescence was monitored at 520 nm, with excitation at 495 nm. After an initial stirring phase of no less than 200 s, 3-6 μl peptide in buffer solution was added. After the fluorescence intensity reached a plateau 50 μl of TRITON-X were added. Fluorescence intensity measurements were performed on a Fluorolog-3 spectrofluorimeter (model FL3–11; JobinYvon Inc., Edison, NJ, USA) equipped with a xenon-arc lamp. See the SI Appendix for further details. *Confocal Microscopy:* GUVs labeled with DiD were prepared for confocal microscopy using electroformation in a 300 mOsm/l sucrose solution. Prepared GUVs were diluted with a glucose buffer (9 mM HEPES, pH 7.40 (KOH), 90 mM KCl, 90 mM EDTA, 120 mM glucose, 300 mOsm/l, filtrated) with 20 μl 50 nM Atto 488 to a total volume of 300 μl. Images were recorded using Olympus IX81 laser scanning confocal microscope (Olympus, Hamburg, Germany). For further details see the SI Appendix. *Cryoelectron Microscopy:* For cryo-EM sample preparation, 4 μl of the sample were applied to plasma-cleaned EM grids (400 mesh copper grids, covered with Quantifoil film (R1.2/1.3)). Samples were plunge-frozen on the grids in liquid

Ethane in a Grid Plunger (Leica EM GP, Leica Microsystems GmbH) with the following parameters: pre-blotting exposure 5 s, blotting time 1.7 s, no post-blotting exposure. Chamber humidity was set to 95% at 22 °C. The LUV solution was treated with R_9 (c = 25 mM) in a ratio of 10:1 for t >60 s immediately before plunge freezing. Cryo-electron micrographs were collected on a JEM-2100F (JEOL Germany GmbH) operated at 200 kV (see the SI Appendix for further details).

Cell Experiments. Forty thousand HeLa cells were seeded to a well of μ-slide (ibiTreat; ibidi, Germany) 16-20 hours before the experiment. Cells were washed with SF-DMEM and kept at 4 °C for 15 min to inhibit endocytic processes. For treatment, pre-cooled (4 °C) 15 μM solution of a peptide in SF-DMEM was added to cells via media exchange and incubated for indicated periods of time at 4 °C. In selected cases, cells were treated for 3 min with a peptide at 4 °C, washed with pre-cooled SF-DMEM and further incubated for indicated period of time at 4 °C in fresh SF-DMEM. Cells were imaged using scanning confocal microscope (FluoView 1000, Olympus) and the tomograms we acquired on Titan Halo transmission electron microscope (see the SI Appendix for details).

Computational Details. We use all atom molecular dynamics (MD) for the fusion process: In a first setup, we created two curved membranes via lipid population imbalances at the two leaflets of each bilayer. In the second setup, we put a very small vesicle composed of in the unit cell and let it fuse with its periodic image. Both of these approaches facilitate formation of the stalk without enforcing its shape. For calcium fusion we used optimized charge-scaled force fields for ions, to account effectively for electronic polarization effects. For vesicle aggregation and bifurcation calculations we employed coarse-graining methods. See the SI Appendix for full details.

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