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Tunable Membrane Potential Reconstituted in Giant Vesicles Promotes Permeation of Cationic Peptides at Nanomolar Concentrations

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Abstract

We investigate the influence of membrane potential on the permeation of cationic peptides. Therefore, we employ a microfluidic chip capable of capturing giant unilamellar vesicles (GUVs) in physical traps and fast exchange of chemical compounds. Control experiments with calcein proved that the vesicle membranes' integrity is not affected by the physical traps and applied shear forces. Combined with fluorescence correlation spectroscopy, permeation of fluorescently labeled peptides across vesicle membranes can be measured down to the nanomolar level. With the addition of a lipophilic ruthenium(II) complex Ru(C17)₂²⁺, GUVs consisting of mixed acyl phospholipids are prepared with a negative membrane potential, resembling the membrane asymmetry in cells. The membrane potential serves as a driving force for the permeation of cationic cell-penetrating peptides (CPPs) nonaarginine (Arg9) and the human immunodeficiency virus trans-activator of transcription (TAT) peptide already at nanomolar doses. Hyperpolarization of the membrane by photo-oxidation of Ru(C17)₂²⁺ enhances permeation significantly from 55 to 78% for Arg9. This specific enhancement for Arg9 (cf. TAT) is ascribed to the higher affinity of the arginines to the phosphoserine head groups. On the other hand, permeation is decreased by introducing an additional negative charge in close proximity to the N-terminal arginine residue when changing the fluorophore. In short, with the capability to reconstitute membrane potential as well as shear stress, our system is a suitable platform for modeling the membrane permeability of

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Author Contributions

C.-C.L. and P.S.D. designed the research. C.-C.L. performed the experiments and analyzed the data. M.B. and K.V. synthesized and characterized the ruthenium complex. S.B. designed and fabricated the microfluidic devices. All the authors have given approval to the final version of the manuscript.

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pharmaceutics candidates. The results also highlight the membrane potential as a major cause of discrepancies between vesicular and cellular studies on CPP permeation.

Keywords

cell-penetrating peptides; membrane permeation; membrane potential; microfluidics; fluorescence correlation spectroscopy

Introduction

Cell-penetrating peptides (CPPs) comprising 5–40 amino acids have attracted considerable research interest due to their potential as carriers to deliver otherwise membrane-impermeable proteins, small-molecule drugs, oligonucleotides, fluorophores, and magnetic resonance imaging agents.^{1–3} The most renowned examples are the TAT peptide (9–13 residues), which is the truncated basic domain of the transactivator of transcription protein from human immunodeficiency virus (HIV),^{4–6} and penetratin (16 residues), derived from the homeodomain of *Drosophila antennapedia*.^{7,8} Both example peptides are enriched in cationic residues, namely, arginines or lysines, which are characteristic of CPPs. Different permeation mechanisms have been proposed^{9,10} and can be categorized into (i) active endocytosis and (ii) passive direct translocation, the latter becoming predominant at higher concentrations of the peptides.^{11,12} Nonetheless, it has been shown that arginines facilitate cellular uptake more than lysines due to the formation of bidentate hydrogen bonds with negatively charged phosphate, carboxylate, or sulfate groups on cell membranes.^{13,14} As a result, synthetic polyarginines (typically 6–12 residues) have also been widely explored.^{15,16}

Model systems have been developed to study membrane translocation. The CPPs are often conjugated to fluorophores to trace the cellular uptake.^{11–14} Although the conjugation may alter properties of the peptides due to the hydrophobicity of the fluorophores, it can serve to emulate the behavior of the CPP–small-molecule cargo. On the other hand, artificial phospholipid membranes have been used as simplistic cell membrane models to delineate peptide–membrane interactions. These include highly curved large unilamellar vesicles^{17,18} with diameters between 100 nm and 1 μm and giant unilamellar vesicles (GUVs), which are larger than 1 μm and thus resemble the cell geometry.^{19–21} These model membranes enable systematic investigation of lipid compositions, which influence the affinity to the peptides. Besides electrostatic interactions between cationic CPPs and the lipid head groups, it has been shown that the membrane potential plays a critical role in CPP permeation.^{17,18,22,23}

In this work, we employ fluorescence correlation spectroscopy (FCS) to probe down to the nanomolar level the permeation of Atto488-conjugated nonaarginine (Arg9-Atto488) and Atto488 as well as Alexa488-conjugated HIV-TAT peptides (TAT-Atto488 and TAT-Alexa488) through GUV membranes. Besides single-molecule sensitivity, there are several advantages of the FCS technique over measurements of fluorescence intensities (e.g., imaging) such as (i) the capability to infer molecular properties, such as aggregation; (ii) the obtained concentration is free from the effects of surrounding solutions, which can otherwise bias the quantification while taking only the intensities;²⁰ and (iii) with confocal detection,

the interference of out-of-focus fluorescence from molecules adsorbed onto membranes and surfaces is minimized. Furthermore, with the external addition of a dialkylated tris(bipyridine)ruthenium(II) complex $\text{Ru}(\text{C17})_2^{2+}$ to the GUV membrane (Scheme 1a), we are able to create an artificial membrane potential to drive permeation. When $\text{Ru}(\text{C17})_2^{2+}$ is photo-oxidized in the presence of potassium ferricyanide (Scheme 1b), the membrane hyperpolarizes and the driving force increases.²⁴ To capture and inspect individual GUVs (Figure 1a), we utilize a microfluidic device with 60 hydrodynamic traps and individual donut-shaped valves.^{25,26} The device not only allows us to map out the correlation between permeation and vesicle size but also permits fast compound exchange as well as measurements under an external flow.

Experimental Section

Synthesis and Characterization of $\text{Ru}(\text{C17})_2^{2+}$

The dihepta-decylated tris(bipyridine)ruthenium(II) complex was synthesized according to the reported procedure²⁷ and is elaborated in the Supporting Information, together with the ^1H NMR data. In brief, the diheptadecylated ligand was first synthesized from 4,4'-dimethyl-2,2'-bipyridine and hexadecyl bromide. $\text{Ru}(\text{C17})_2^{2+}$ was formed following the treatment of the diheptadecylated ligand with $\text{Ru}(\text{bpy})_2\text{Cl}_2$ by refluxing ethanol. The final complex was confirmed by ^1H NMR and mass spectrometry. The experimentally determined mass for the product was found to be $m/z = 523.3$ [M^{2+}].

GUV Preparation

The GUVs were produced by the water-in-oil emulsion transfer method.^{28,29} The zwitterionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and mono-anionic 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) were purchased from Avanti Polar Lipids and the mineral oil (grade suitable for infrared spectroscopy, light oil) from Sigma-Aldrich. A mineral oil solution containing 160 μM POPC and 40 μM POPS was first prepared, 500 μL of which was then deposited on top of a 50 μL aqueous “droplet solution” (1 M sucrose, 140 mM KCl, 10 mM *N*-(2-hydroxyethyl)-piperazine-*N'*-ethanesulfonic acid (HEPES), pH 7.4) and 200 μL on top of a 500 μL aqueous “hosting solution” (1 M glucose, 140 mM NaCl, 10 mM HEPES, pH 7.4). Following agitation of the droplet solution, a water-in-oil emulsion was formed and transferred to the hosting solution tube. Subsequently, it was centrifuged at 500g for 10 min. After washing of the pellet, the GUVs were resuspended in 150 μL hosting solution, and the final lipid concentrations were determined to be ~ 100 μM via the Bartlett assay.³⁰

To create the negative membrane potential, an aqueous stock solution of 200 μM $\text{Ru}(\text{C17})_2^{2+}$ was prepared with 0.2% dimethyl sulfoxide (DMSO), which was later diluted to 1:20 into GUV solutions to achieve a final concentration of 10 μM .²⁴ Typical incubation time was 45 min. For monitoring the effect of $\text{Ru}(\text{C17})_2^{2+}$ on GUV morphologies, 2.5 μL of 2 mM calcein disodium salt (Fluka) stock solution was added to the droplet solution, yielding a final concentration of 100 μM to be encapsulated.

ζ -Potentials of the GUVs containing 1 M sucrose were measured in 1 M glucose with the Zetasizer Nano ZSP (Malvern). The ζ -potential measurements taken up to 9 h after incubation with $\text{Ru}(\text{C17})_2^{2+}$ imply that the generated membrane potential was maintained throughout the experimental period. There was only a slight shift in the ζ -potential from -11.7 ± 4.8 to -18.5 ± 2.6 mV,³¹ whereas that of pure GUVs with 20% POPS was found to be -90.8 ± 3.3 mV.

Microfluidic Device Preparation

The master wafers and poly(dimethylsiloxane) chips were fabricated as previously described^{25,26} and detailed in the Supporting Information. In short, the device comprised a top pressure layer and a bottom fluid layer 20 μm in height and was finally bonded to a cover glass (#1.5, Menzel-Gläser). All the solutions were infused into the fluid layer using a syringe pump (NanoJet, Chemyx). The channels were coated with 4% (w/v) bovine serum albumin (BSA) (Sigma-Aldrich) in phosphatebuffered saline buffer (pH 7.4, 1 $\times$, Gibco) before GUVs were introduced and trapped. Afterward, the pressure valves were activated through a home-built pressure controller to 2 bar, whereas 20 nM calcein, 10–35 nM Arg9-Atto488, TAT(RRRQRRKKRG)-Atto488, TAT-Alexa488 (PSL Peptide Specialty Laboratories; all labels attached to the N-terminal arginines and purified as trifluoroacetate salts), or 50 nM FAM-Adp₈, FAM-(AdpOMe)₈, FAM-(AdpNMe₂)₈ (synthesized with fluorenylmethyloxycarbonyl (Fmoc) chemistry, as published in ref 32) in the hosting buffer was flushed into the channels. The integrated valves were opened and closed again for FCS measurements, except for the experimental series under flow. For the photo-oxidation reaction, a 20 mM stock solution of potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$, Acros) was diluted 1:100 into the peptide solution to reach a final concentration of 200 μM .

GUV Imaging

The microscopy setup was based on an Olympus IX71 inverted microscope equipped with a water immersion objective (UPlanSApo 60 $\times$ /1.20 W, Olympus), a Lumencor Spectra X LED light engine and a UK1117-M CCD camera (ABS). To image both the GUV membrane incorporating $\text{Ru}(\text{C17})_2^{2+}$ (emission spectrum displayed in Figure 1b) and the GUV lumen loaded with calcein, a cyan light-emitting diode (LED) with an excitation filter 474/27 BrightLine HC (Semrock) served as the excitation source. The dichroic mirror and emission filter combinations used for $\text{Ru}(\text{C17})_2^{2+}$ and calcein, respectively, were 494 LP plus E590lpv2 (Chroma) and 409/493/573/652 HC quadband BS plus 432/515/595/730 HC quadband filter (Semrock). The size of each GUV was measured at the z-position, where the diameter appeared to be largest, and was averaged over *x*- and *y*-directions.

Fluorescence Correlation Spectroscopy (FCS)

Output from a 488 nm diode laser (Cobolt MLD, Cobolt) was expanded to a beam diameter of 5 mm, corresponding to 69.4% underfilling of the objective back aperture,³³ and then coupled into the microscope at an attenuated power of 60 μW . The fluorescence was passed through an emission bandpass filter (525/50 BrightLine HC, Semrock) and focused onto a multimode fiber with 50 μm core diameter (FG050LGA, Thorlabs), which was connected to an avalanche photodiode (SPCM-CD 3017, PerkinElmer). Measurements were started after the GUVs had been exposed to the peptides/molecules for 30 min.

Fluorescence signals were correlated and recorded by the ALV-5000/60X0 Multiple Tau Digital Correlator and the accompanying ALV-Correlator Software (Version 3.0). Subsequently, the autocorrelation curves were fitted using QuickFit software,³⁴ taking into account the triplet state (the first bracket)

$$G(\tau) = \left[1 + \frac{\Theta_{\text{trip}}}{1 - \Theta_{\text{trip}}} e^{-\tau/\tau_{\text{trip}}} \right] \left[\frac{1}{N} \frac{1}{1 + \tau/\tau_D} \frac{1}{\sqrt{1 + (r_0/z_0)^2(\tau/\tau_D)}} \right] \quad (1)$$

where N is the average number of molecules in a three-dimensional Gaussian-approximated focal volume, with radial and axial widths of r_0 and z_0 . τ_D and τ_{trip} denote the fitted diffusion time and triplet decay time, respectively, whereas Θ_{trip} stands for the triplet fraction. Average molecular brightness is obtained by dividing the average count rate with N . Together with τ_D , it serves as the criteria for distinguishing low concentration (down to 0.03 nM) from the background. For flow rates up to 11 $\mu\text{L}/\text{min}$, the autocorrelation curves were still well-fitted by the model and the amplitude of the diffusion component ($1/N$ in the second bracket) was not affected.³⁵ Uncorrelated background from the buffer was accounted for in the calculations of all N .³⁶

Results and Discussion

Ru(C17)₂²⁺ Does Not Alter the Membrane Permeability

To validate the method and test the effect of Ru(C17)₂²⁺ on the vesicle membranes, we measured first the permeation of calcein, a commonly used cytosol stain, into Ru(C17)₂²⁺-incorporating GUVs. Translocation of calcein into GUVs was measured by comparing the average number of molecules inside, N_{in} , and outside, N_{out} , the vesicle. The average number of calcein molecules was measured by positioning the laser focus inside or outside a trapped GUV (exemplified in Figure 1c) and measuring 10 times for 10 s. The permeation is defined as $N_{\text{in}}/N_{\text{out}}$. For Ru(C17)₂²⁺-incorporating GUVs, the average permeation of 14% (Figure 2a) was similar to that of GUVs without Ru(C17)₂²⁺, which yielded 17% (Figure 2b, black open squares). Measurements were repeated using GUVs consisting of 100% POPC, in which the electrostatic repulsion between calcein and the phosphoserine head groups was excluded (Figure S1, black open squares). Comparable results indicate that neither electrostatic interaction nor membrane potential has a noticeable impact on the permeation of such a xanthene-based molecule with acidic functional groups. More importantly, the equally low permeation levels of the small molecule calcein with and without Ru(C17)₂²⁺ also demonstrates that the incorporation of Ru(C17)₂²⁺ does not cause leakiness of the membrane. The reciprocal relationship between the concentration and the correlation amplitude enables us to probe low permeation levels and consequently sub-nanomolar concentrations inside the GUVs with the aid of diffusion time and molecular brightness parameters.

Permeation of Calcein is Dependent on Vesicle Size

With the microfluidic device, individual GUVs were analyzed and hence additional parameters can be correlated to the permeation levels. Notably, the distribution of calcein permeation in the absence of $\text{Ru}(\text{C17})_2^{2+}$ was highly asymmetric (Figure 2b). As vesicle diameter decreases from 20 μm down to 10 μm , permeation increased significantly from below 10 to 60%, which might be caused by different amount of residual oil between the bilayer. At sub-20 μm diameters, addition of $\text{Ru}(\text{C17})_2^{2+}$ and hence the creation of a negative membrane potential (unfavorable to the permeation of anionic compounds like calcein) suppressed the permeation to always below 30% (Figure 2a).

Meanwhile, we recognized that the monitored GUVs were on average larger without $\text{Ru}(\text{C17})_2^{2+}$ than after incubation with $\text{Ru}(\text{C17})_2^{2+}$. Therefore, we pooled all the samples together and plotted the size distribution histograms (Figure 3, including those described in the following sections). Indeed, the vesicle diameter decreased from 23.0 ± 6.6 to 17.9 ± 4.8 nm upon the incorporation of $\text{Ru}(\text{C17})_2^{2+}$ (or further to 15.8 ± 3.8 nm with the addition of potassium ferricyanide). The size reduction may be attributed to the higher membrane curvature induced by $\text{Ru}(\text{C17})_2^{2+}$, as alkylated ruthenium(II) bipyridyl complexes are known to form micelles.²⁷ On the other hand, GUVs have been demonstrated to incur various types of morphological changes upon addition of amphiphilic molecules.^{37,38} Thus, we prepared the GUVs loaded with calcein to visualize such effects. The vesicles were incubated off-chip with 10 μM $\text{Ru}(\text{C17})_2^{2+}$ for 15 min and then transferred to a cover glass-sandwiched flow channel³⁹ coated with BSA. Membrane evagination was observed, followed by detachment of a small membrane portion leading to shrinkage of the parent vesicle (Figure S2).

Permeation of Calcein is Independent of Flow

The vesicle traps employed here also allow us to investigate whether shear force in the microfluidic channels alters membrane permeability. Although shear force can be sensed by cells through mechanotransduction pathways, which involve multiple components such as ion channels and membrane receptors,⁴⁰ it has been found that membrane fluidity and lipid order are also directly modified.^{41,42} To this end, we monitored the effect of such increased membrane fluidity on the permeation of calcein by infusing it at nominal flow rates of 2, 5, 8, and 11 $\mu\text{L}/\text{min}$. The results for GUVs containing 20% POPS are summarized in Figure 2b (solid symbols) and those for GUVs with 100% POPC are given in Figure S1. Analogous to static conditions, the permeation remained below 10% for larger vesicles and increased monotonously when the vesicle size decreased below 20 μm . This suggests that surface area and/or curvature are more critical to the permeation of such small molecules, and that the applied shear force does not induce higher membrane permeability.^{43,44}

Membrane Potential Facilitates Permeation of Cationic Peptides

We have previously demonstrated that octaarginine derivatives, which penetrated into HEK293 cells, did not permeate at micromolar concentrations into the GUVs bearing 10% negatively charged lipids.¹⁹ They accumulated only in the membrane, as it was the case with apoptotic cells in which membrane asymmetry was lost and phosphatidylserine became exposed.^{45,46} This implies that although negatively charged lipids recruit cationic CPPs, a negative membrane potential is necessary for the CPPs to finally translocate. With GUVs

carrying 20% POPS, we did not observe autocorrelation curves that could be fitted with proper diffusion times and brightness, signifying no permeation (Figure S3).

After incubation with $\text{Ru}(\text{C17})_2^{2+}$, clear autocorrelation curves were recorded inside the GUVs (Figure 1c). The permeation of Arg9-Atto488 into individual vesicles is displayed in Figure 4 (blue open squares) and the average permeation was found to be 55, 49, and 42% for Arg9-Atto488, TAT-Atto488, and TAT-Alexa488, respectively (Figure 5a–c, blue), all of which are significantly higher than that for calcein (14%, Figure 5d). This indicates that passive translocation of the CPPs is already taking place at the nanomolar level. The amount of $\text{Ru}(\text{C17})_2^{2+}$ incorporated into the membranes was analyzed by comparing the concentration remaining in the supernatant (extinction coefficient $\epsilon = 15\,700\text{ M}^{-1}\text{ cm}^{-1}$ at 457 nm, Figure 1b), following incubation and centrifugation, to the control without GUVs. Assuming that $\text{Ru}(\text{C17})_2^{2+}$ is only added to the outer leaflet of the lipid bilayer, the complex-to-lipid ratio is estimated to be $12.9 \pm 3.0\%$ and the negative charges in the outer leaflet contributed by 20% POPS were essentially neutralized (Scheme 1a).

We were able to induce membrane hyperpolarization²⁴ by illuminating the $\text{Ru}(\text{C17})_2^{2+}$ -stained GUVs for 15 s (20% LED intensity) after introducing 200 μM potassium ferricyanide together with the peptides into the chambers. The advantages of using of $\text{Ru}(\text{C17})_2^{2+}$ are (i) the large Stokes shift, minimizing its interference onto the green detection channel (Figure 1b) and (ii) the possibility to carry out photoreactions due to its long excited-state lifetime.⁴⁷ For Arg9-Atto488, the permeation was enhanced significantly to 78% (Figure 4, solid magenta circles and Figure 5a, magenta), whereas the permeation remained statistically unchanged at 61 and 34% for the TAT peptides (Figure 5b,c, magenta). The difference can be attributed to the higher affinity of arginine to the phosphoserine head groups. Upon photo-oxidation, the electrostatic repulsion between $\text{Ru}(\text{C17})_2^{2+}$ and the peptides was increased in addition to increase in membrane potential. Therefore, the peptides originally bound to the membrane would either translocate through the membrane or be repelled from the surface. With its stronger affinity to the membrane, more Arg9 remained bound and could then translocate.

Variation in the fluorescent label from Atto488 to Alexa488 showed that the introduction of an additional negative charge (a carboxylate substituent on the phenyl ring) in close proximity to (cf. a C4 linker in Atto488) the first arginine residue has a moderate influence on the permeation. The permeation of TAT-Alexa488, compared with that of TAT-Atto488, decreased only slightly from 49 to 42%. However, upon photo-oxidation and the subsequent increase in electrostatic repulsion, the permeation of TAT-Alexa488 (34%) was significantly lower than that of TAT-Atto488 (61%), likely due to the neutralization of the first arginine residue by the negatively charged fluorophore. It was then most easily repelled from the membrane among the three CPPs. This further emphasizes the role of each arginine residue in binding to the membrane.

Compared with the permeation of calcein (Figure 2a), measurements for the potential-driven permeation of CPPs appeared to be more scattered (Figure 4), possibly owing to heterogeneity and residual oil introduced by the bulk GUV production method. However, similarly broad distributions have been observed even for well-controlled droplet interface

bilayers.^{22,23} This may be circumvented by generating the GUVs on-chip.⁴⁸ Nevertheless, contrasts between the permeation of cationic CPPs with and without photo-oxidation versus negative controls in the absence of $\text{Ru}(\text{C17})_2^{2+}$ clearly indicate that the negative membrane potential is essential.

Oligomeric β -Dipeptides Do Not Permeate Regardless of the Membrane Potential

As a comparison, permeation of the carboxyfluorescein(FAM)-labeled octa-Adp (FAM-Adp₈), derived from the biopolymer cyanophycin (found in cyanobacteria and blue-green algae as a nitrogen and carbon storage material),⁴⁹ was measured. It comprises eight aspartates as the backbone, with each side chain connected to an arginine, rendering the molecule neutral at pH 7.4. Similar to the CPPs, in the absence of $\text{Ru}(\text{C17})_2^{2+}$, no permeation was observed (Figure 5d, gray). With $\text{Ru}(\text{C17})_2^{2+}$, half of the vesicles showed no permeation, whereas the other half averaged 12% (Figure 5e, blue), yielding an asymmetric distribution and an overall average permeation of 7% (singles data points are displayed in Figure S4). The results confirm that the negative membrane potential promotes exclusively the permeation of cationic peptides.

When the free carboxylic acid groups on the side chains of FAM-Adp₈ were replaced by methyl-ester (FAM-(AdpOMe)₈) or dimethyl-amide (FAM-(AdpNMe₂)₈) groups, the molecule bears again eight positive charges. Surprisingly, neither derivatives showed permeation both in the absence and presence of $\text{Ru}(\text{C17})_2^{2+}$ (data not shown because no proper autocorrelation curves were recorded inside the GUVs). This corroborates our recent findings in ref 50 that none of these three molecules permeated into HEK293 cells at the concentration of 2 μM . The fact that the cationic ester and amide derivatives entered only into MCF-7 cells but not HEK293 cells or GUVs with a reconstituted membrane potential suggests that the permeation is driven by active endocytosis and may be specific to cancer cells.⁵¹

The observation that linear CPPs permeated under a membrane potential but the cationic octa-Adp derivatives did not can be rationalized by both the cross section and the amphiphilicity of the molecules. The critical size for passive translocation through the membrane may fall between an α -peptide and a β -dipeptide. Alternatively, we speculate that for the octa-Adp derivatives, the relatively hydrophobic backbone may be better shielded by the arginine guanidinium side chains and thus cannot interact with the acyl chains in the membrane bilayer.

Conclusions

In this work, we made GUVs in physiological buffers mimicking intra- as well as extracellular environments, which is not attainable with the conventional electroformation method.⁵² Moreover, the membrane potential was reconstituted by adding the diheptadecylatedbipyridine bisbipyridine ruthenium(II) complex externally. This triggers the translocation of fluorescently labeled polybasic peptides, which does not take place even when adsorbed onto the membrane via electrostatic interactions. With the single-molecule sensitive FCS technique, we revealed that such passive translocation occurs already at concentrations down to 10 nM, which is favorable for drug delivery, as the cargo would end

up in the cytosol instead of the endosome. The different behavior of Arg9 and TAT peptides following photo-oxidation and hence membrane hyperpolarization corroborates a two-step entry mechanism. First, the peptides are bound to the membrane via interaction with the phosphoserine head groups, after which the negative membrane potential facilitates translocation. The bulkiness of the $\text{Ru}(\text{C17})_2^{2+}$ bipyridine ligands ensure the availability of the phosphoserine carboxylates for hydrogen bonding, despite overall charge neutralization by ruthenium-(II). For Arg9, the binding affinity is stronger so that it withstands repulsion from the photo-oxidized ruthenium(III) complex and senses the enhanced driving force, resulting in a higher permeation following hyperpolarization. In the case of cell membranes, in which the anionic lipids are predominantly located in the inner leaflet,⁴⁶ glycosaminoglycans such as heparan sulfate would then serve as binding partners.⁵³

Our study also highlights the influence of the fluorescent label, which has to be considered in future studies. Attachment of a negatively charged fluorophore or organic drug molecule directly through an amide bond to a basic residue may impair the affinity of the short CPP to the membrane. Moreover, comparison between the linear CPPs and the β -dipeptide octa-Adp derivatives underlines the limits in molecular cross section and amphiphilicity for passive translocation through the membrane. The dimensions of GUVs, which resemble that of a cell, will allow us to screen the permeation of CPPs conjugated not only to small molecules but also to nanoparticles or liposomes¹ and characterize their interactions with individual membrane components. Our microfluidic approach of vesicle trapping also enables us to keep track of the additional parameters, such the vesicle size or shear force, simultaneously.

To sum up, combining microfluidics, fluorescence correlation spectroscopy, and giant unilamellar vesicles as a membrane model, we were able to verify the importance of membrane potential to the permeation of polycationic cell-penetrating peptides. With this platform, we can test the effect of individual membrane components on the permeation of CPP-payload conjugates at low doses down to the nanomolar level.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

GUV trapping, imaging, and FCS measurements. (a) A GUV stained with $\text{Ru}(\text{C17})_2^{2+}$ was captured in the hydrodynamic trap, illustrated in light blue. Scale bar: $10\ \mu\text{m}$. (b) Extinction coefficient ϵ (black line) and emission spectrum of $\text{Ru}(\text{C17})_2^{2+}$ (red line) measured in water with 0.01% dimethyl sulfoxide (DMSO). The green shaded area indicates the transmission profile of the bandpass filter for FCS measurements. (c) Representative FCS measurements of Arg9-Atto488 inside (black open circles) and outside (blue open triangles) of a GUV incorporating $\text{Ru}(\text{C17})_2^{2+}$. The red and magenta curves are the respective diffusion components obtained from eq 1.

Figure 2.

Correlation between the permeation of calcein into individual GUVs and their sizes. (a) Permeation into GUVs incorporating Ru(C17)_2^{2+} and under static conditions. (b) Permeation into GUVs without Ru(C17)_2^{2+} and measured under static conditions (black open squares) as well as in the specified flow rates.

Figure 3. Size-distribution histograms of all GUVs measured (a) in the absence Ru(C17)_2^{2+} , (b) with Ru(C17)_2^{2+} , and (c) with Ru(C17)_2^{2+} plus potassium ferricyanide.

Figure 4. Permeation of Arg9-Atto488 measured for individual GUVs incorporating Ru(C17)_2^{2+} with (solid magenta circles) and without (blue open squares) 200 μM potassium ferricyanide.

Figure 5.

Permeation of Arg9-Atto488 (a), TAT-Atto488 (b), TAT-Alexa488 (c), calcein (d), and FAM-Adp8 (e) into GUVs consisting of 20% POPS and 80% POPC. The y-axis represents N_{in}/N_{out} . Solid symbols in each column denote the mean values, boxes the 25/75 percentiles, the middle line the medians, and whiskers the standard deviations. Gray boxes indicate measurements in the absence of $\text{Ru}(\text{C17})_2^{2+}$. Blue boxes indicate measurements with $10 \mu\text{M}$ $\text{Ru}(\text{C17})_2^{2+}$ and magenta boxes additionally with $200 \mu\text{M}$ potassium ferricyanide. Asterisks (***) designate when the data sets are statistically different ($P < 0.001$, two-tailed unpaired t-test), whereas $P > 0.05$ for ns. The number of GUVs measured in each condition ranged from 21 to 41.

Scheme 1. Ru(C17)_2^{2+} to Create a Tunable Membrane Potential^a

^a(a) Negative membrane potential created on a GUV via addition of Ru(C17)_2^{2+} promotes permeation of cationic CPPs. (b) Structure of Ru(C17)_2^{2+} and the photo-oxidation reaction.