Bacterial Cell Penetration by β^3-Oligohomoarginines: Indications for Passive Transfer through the Lipid Bilayer

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Recent studies on the mechanisms of uptake of cell-penetrating peptides (CPPs)[1] by mammalian cells provide evidence that one possible pathway for peptide entry involves initial cell-surface binding of the peptide carrying positively charged side chains followed by endocytosis and cytoplasmic trafficking.[2,3] This endocytotic uptake mechanism has been verified with various techniques, such as fluorescent peptide probes, flow cytometry, and confocal laser scanning microscopy (CLSM).[2,4,5] However, we have also become aware of some reports showing that various putative endocytosis inhibitors, low temperature, or cell-energy-depletion conditions could not effectively suppress peptide uptake; this suggests a passive, direct transfer through the plasma membrane.[5–9] With regard to these findings, it is noteworthy that counteranions and the electric potential across the biological membrane play an important role in the cell penetration of polycationic compounds.[9] Although an increasing number of reports have appeared, in which the occurrence of misleading artifacts due to cell fixation or fluorescence bound to the cell surface have been described,[4,10–12] the existence of alternative transport mechanisms can not be excluded.[13] Such pathways will have significant implications for understanding the fundamental functions of biological membranes as well as for designing novel, medicinally valuable peptide–drug conjugates based upon CPPs as molecular transporting vehicles.[1] The goal at the outset of the present study was to shed new light on alternative uptake pathways. To this end, we incubated our fluorescently labeled CPPs comprised of β^3-homoarginine with selected strains of bacteria that lack the normally indigenous endocytotic mechanism associated with mammalian cells. Little was known about the uptake of CPPs by microorganisms until recently, when it was reported that CPPs improve drug delivery into bacteria and fungi.[14–16]

Our experiments were specifically designed to avoid potential artifacts by cell fixation and to discriminate the internalized portion of peptide from the extracellular surface-bound portion. Thus, in addition to conventional fluorescence microscopic techniques, we employed confocal laser scanning microscopy (CLSM) to gain more precise and direct information on the peptide distribution within unfixed cells. This approach was further complemented with spectrofluorometric assays by using NBD-labeled β-oligoarginines as quenchable fluorescent probes (Figure 1). The fluorescent dye 7-nitrobenzo-2-oxa-1,3-

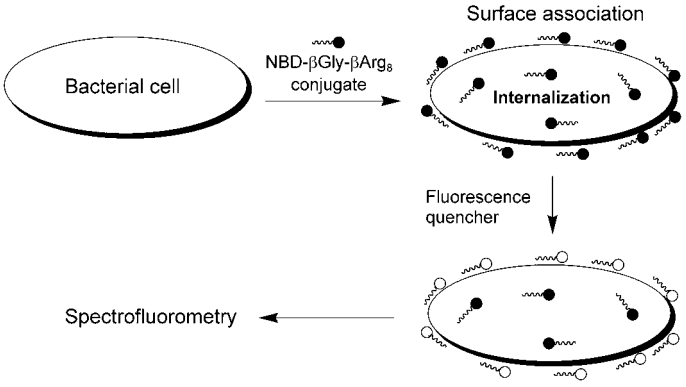

Figure 1. Quenching principle of the surface-associated fluorescence from NBD-labeled β-octaarginines by quenching agents. The uptake rate can be determined by comparing the initial fluorescence with the fluorescence after addition of quenching agents. NBD-labeled β-octaarginines (fluorescent) ●~~, quenched NBD-labeled β-octaarginines (nonfluorescent) ○~~.

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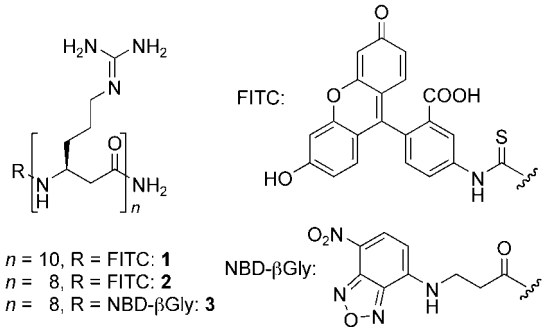
diazol (NBD) can be quenched by different agents. Provided that these reagents do not cross the cell membrane, it is a simple method to measure the transbilayer distribution of NBD-labeled peptides by comparing the fluorescence intensity before and after the addition of quenching agents.[12]

Based on their proven ability to cross the phospholipid bilayer of mammalian cells, fluorescein (FITC)-labeled β-deca- and β-octaarginine amides (Scheme 1; **1** and **2**) were selected as the fluorescent probes for microscopic observation.[17,18] In a previous study, we demonstrated that these substances are neither hemolytic towards rat and human erythrocytes nor do they inhibit the growth of six different bacterial strains.[17] The peptide was prepared on Rink Amide AM resin according to the previously described method.[17] In addition, NBD-labeled β-octaarginine amide (**3**) was prepared specifically for use in experiments designed to distinguish the internalized peptide from the surface-bound peptide with the help of a membrane-impermeable quenching agent. The synthesis of the peptide was carried out by starting from resin-bound β-octaarginine[17] and NBD-β-homoglycine (**4**)[19] (Scheme 2). *Escherichia coli* was chosen as the best-known representative of Gram-negative bacteria, whereas *Bacillus megaterium* was chosen as a Gram-positive model organism because of its relatively large size suitable for microscopic observations. Both strains were grown in nutrient broth at 30 °C until an optical density (OD_{550}) of 2.6–3.6 was reached. Prior to the experiments, the cell suspensions were centrifuged, the pelleted cells were washed once and then resuspended in phosphate-buffered saline (PBS; see the Supporting Information for more details).



Scheme 1. Fluorescently labeled peptides composed of β^3-oligoarginine amides used for the uptake studies.

We first checked our uptake conditions with the two strains using a conventional fluorescence microscope. Both strains showed fluorescence after incubation with FITC-labeled β-decaarginine amide **1** (Figure 2) while no fluorescence was observed when the cells were incubated with free FITC under the same experimental conditions (not shown).

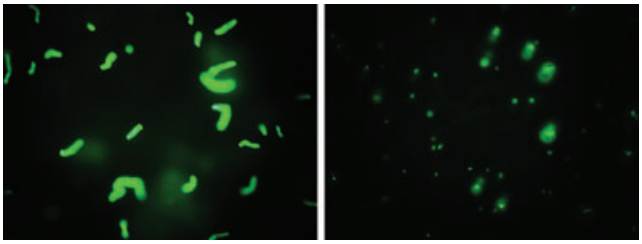
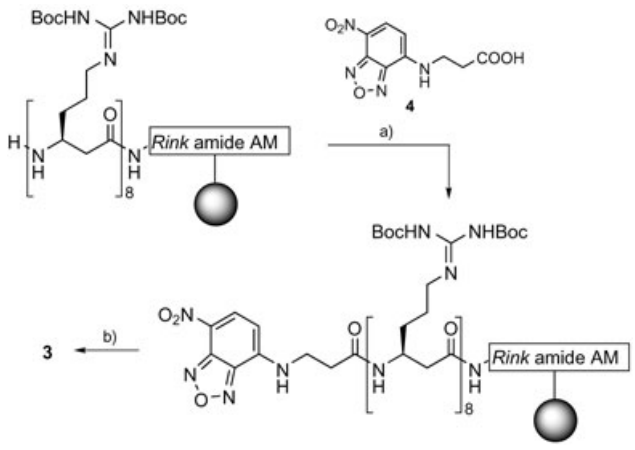


Figure 2. Microscopic analysis of FITC-labeled β-decaarginine amide internalization by *B. megaterium* (left) and *E. coli* cells (right). After 15 min incubation with the peptide (5 μM), cells were washed and monitored by fluorescence microscopy (total magnification 600×).

Next, we employed two further methods to prove that the polycationic β-peptides were transported into the cells, and that the fluorescence was not only due to the β-peptide bound to the negatively charged bacterial cell wall. It was possible to monitor different layers of a single *B. megaterium* cell by CLSM; a large spread of fluorescence within the cytoplasm was observed after incubation with FITC-labeled β-decaarginine amide **1** (Figure 3 A). Again the control with free FITC did not exhibit any fluorescence (not shown). Moreover, cell-surface staining by wheat germ agglutinin conjugated to Alexa® Fluor 633 clearly indicated that the FITC-labeled peptide **2** was associated with the cytoplasm and not with the Gram-positive bacterial cell wall (Figure 3 B).[20,21] The homogeneous distribution of cytoplasmic fluorescence is in clear contrast to the distribution of the fluorescence in mammalian cells. There the fluorescence is mainly localized in subcellular organelles, such as endosomes, nuclei, and/or nucleoli, none of which exist within the bacterial cell.

In the NBD-quenching assays, we had observed that the standard procedure with dithionite as the quenching agent did not work for the present system. Although commonly used for NBD quenching,[22] this assay is extremely sensitive towards temperature changes.[12] In previous studies, dithionite reduction of NBD fluorophores did not work in *B. megaterium* systems.[23] We also observed that dithionite evidently passes through the cell membrane of *B. megaterium* and reduces the NBD both inside and outside the cells. Cobalt chloride was suggested as an alternative quenching agent, especially for *Ba-*



Scheme 2. Preparation of NBD-labeled β-octaarginine amide **3**. a) *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, *N*,*N*-diisopropylethylamine, **4**, DMF, 25 °C; b) trifluoroacetic acid/triisopropylsilane/H_2O (95:2.5:2.5), 25 °C.

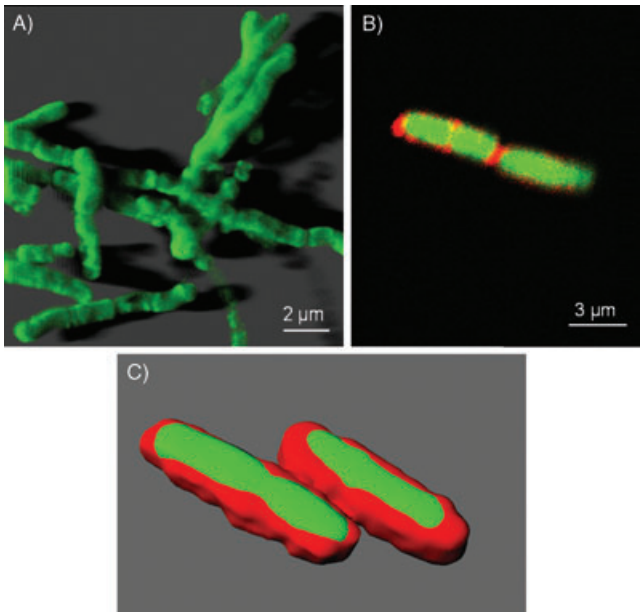


Figure 3. Visualization of the labeled peptide by confocal laser-scanning microscopy. *B. megaterium* cells were incubated with A) 5 μM FITC-labeled β-decaarginine amide and B and C) 5 μM FITC-labeled β-octaarginine amide followed by an incubation step with wheat germ agglutinin conjugated to Alexa® Fluor 633 (a proprietary product of undisclosed structure from Molecular Probes, Inc. with properties similar to long-wavelength Cy fluorochromes). A) 3D reconstruction of serial optical sections. B) One confocal section of a doubly labeled *B. megaterium* cell. The FITC-labeled β-octaarginine (green) is diffusely distributed in the cytoplasm, whereas the signal from the wheat germ agglutinin (red) is concentrated on the cell surface. C) 3D reconstruction of doubly labeled *B. megaterium* cells.

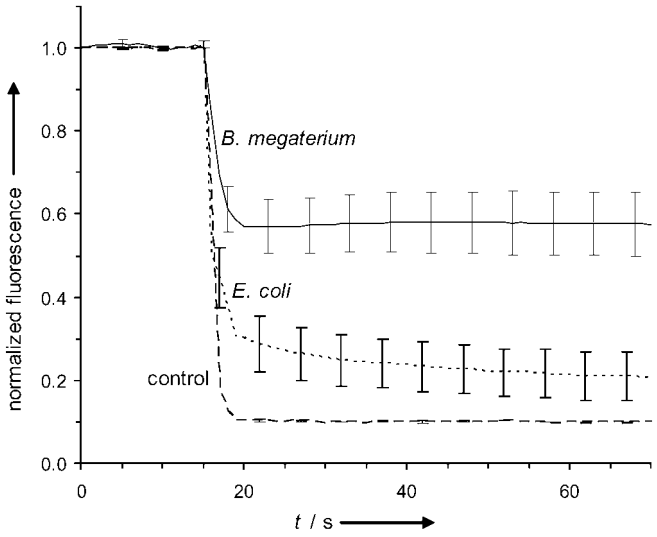


Figure 4. Determination of the fraction of internalized NBD-labeled β-octaarginine amide. Quenching rates of NBD-labeled β-octaarginine associated with *B. megaterium* and *E. coli* cells at 6 °C. The control experiment represents the quenching of NBD-labeled β-octaarginine amide alone in PBS buffer. Each curve is the average of three measurements.

cillus systems,[23] but it did not quench NBD-labeled β-octaarginine amide **3** at all. One reason could be the charge repulsion between Co^{2+} and the positively charged guanidinium groups of the peptide, which could suppress collisional quenching of the excited fluorochrome by Co^{2+}. This was confirmed by preliminary quenching experiments without cells. While the control substance NBD-β-homoglycine was quenched by Co^{2+}, no reduction of fluorescence was observed for NBD-labeled β-octaarginine amide. Finally, addition of surplus potassium iodide enabled the quenching of the cell-bound NBD-labeled β-octaarginine amide **3** (Figure 4).[24] For *B. megaterium*, 42% of the fluorescence was quenched; this indicates that more than half of the apparent fluorescence was protected from collisional quenching by iodide. This value corresponds only qualitatively to the actual amount of peptide that was internalized by the cells because the fluorescence coefficient of NBD is known to vary depending on the lipophilicity of the environment in which the dye resides (e.g. cell membrane or cytoplasm).[25] In addition, self-quenching of NBD fluorescence might become significant when the peptide accumulates within the cell body at high concentrations. In comparison, in the case of *E. coli*, 78% of the fluorescence was quenched. The reduced uptake by *E. coli* might be explained by the different surface/volume ratio of the two bacterial strains, which is approximately twofold higher for *E. coli* than for *B. megaterium* (typical dimensions of *E. coli* and *B. megaterium* are 1×2 μm[26] and 2×5 μm,[27] respectively, with each bacterial cell assumed to have approximately the shape of a cylinder). From the results obtained by these two independent methods, it is unambiguously confirmed that both Gram-positive and Gram-negative bacteria can take up polycationic CPPs into their cytoplasm, as is the case with mammalian cells observed previously in our studies.[17,18] In view of the absence of an indigenous endocytotic mechanism in the prokaryotes, it is now strongly suggested that the observed peptide penetration can be attributed to a pathway other than endocytosis. Whether the same mechanism is involved in mammalian cells is still open to discussion. However, recent work from Wender's group indicates the involvement of a nonendocytotic mechanism in peptide uptake by mammalian cells.[9] Interestingly, at a simpler level of membrane complexity, artificial phospholipid vesicles with negative net surface charge in aqueous solutions have been found to adsorb polycationic peptides on their surface but to be incapable of internalizing them.[17] This is an indication of the presence of as yet unknown fundamental properties uniquely endowed to biological membrane systems for CPP translocation.

In summary, we have demonstrated by two independent techniques that fluorescently labeled CPPs can penetrate into Gram-positive and Gram-negative bacterial strains, and that the uptake mechanism is presumably nonendocytotic. The implication of such an alternative pathway, particularly in the case of mammalian cells, will be assessed and amassed in our laboratory as well as those of others in the relevant fields and will be disclosed in due course.

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