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ABSTRACT: Studies of live cells often require loading of exogenous molecules through the cell membrane; however, effects of loading method on experimental results are poorly understood. Therefore, in this work, we compared three methods for loading a fluorescently-labeled peptide into cells of the model organism *Dictyostelium discoideum*. We optimized loading by pinocytosis, electroporation, and myristoylation to maximize cell viability and characterized loading efficiency, localization, and uniformity. We also determined how loading method affected measurements of enzyme activity on the peptide substrate reporter using capillary electrophoresis. Loading method had a strong effect on the stability and phosphorylation of the peptide. The half-life of the intact peptide in cells was 19 ± 2, 53 ± 15, and 12 ± 1 min, for pinocytosis, electroporation, and myristoylation, respectively. The peptide was only phosphorylated in cells loaded by electroporation. Fluorescence microscopy suggested that the differences between methods were likely due to differences in peptide localization.

The introduction of exogenous molecules into the interior of a cell is a key step in many biochemical and bioanalytical methods. Genetic material, siRNA, antibodies, reporter molecules, drugs, and pro-drugs are a few examples of compounds that may need to be introduced into the cytoplasm. Many of these analytically useful molecules are membrane impermeant. In general, large or hydrophilic molecules cannot cross the cell membrane by passive transport. Consequently, a number of methodologies have been devised to load exogenous molecules into live cells.

Loading methods for exogenous compounds rely on physical, chemical, or biological phenomena to move molecules across the cell membrane, preferably with minimal perturbation of the cell.[1] Microinjection is conceptually straightforward and applicable to a wide range of molecules; however, this method is technically challenging and time consuming, since each cell must be injected individually. Several alternative methods are available to load populations of cells simultaneously. Many cell types undergo pinocytosis, a process in which cells endocytose the surrounding medium. The subsequent application of osmotic pressure can lyse the resulting pinosomes and release their contents into the cytosol.[2] Other methods involve disrupting the cell membrane by electroporation or sonication to allow exogenous molecules to diffuse into the cell through pores in the membrane.[3–5] Exogenous molecules can also be chemically modified to render them membrane permeant. For example, cargo molecules may be conjugated to carrier molecules such as a myristoyl group,[6] cell penetrating peptide,[7] or a caging moiety.[8]

An ideal loading method would result in negligible stress to cells while providing precise control over the concentration and localization of the loaded molecule in cells. In reality, no ideal loading method has been realized, and the methods described above result in varying levels of cell stress, heterogeneous levels of loading between cells, and localization of exogenous molecules to lysosomal compartments or other organelles. Due to these differences in outcomes, it seems likely that the fate of an exogenous molecule will differ depending on the loading method used. Because these differences will affect measurements when molecules are used as reporters or indicators in analytical studies, a thorough understanding of how different loading methods influence the fate of molecular reporters is needed.

In this work, we compare three methods for loading an exogenous peptide substrate reporter into the social amoeba *Dictyostelium discoideum*, a common, single-celled model organism with particular relevance for studies of cell motility, chemotaxis, differentiation, and morphogenesis.[9] The fluorescently labeled peptide used in this study is a reporter substrate for protein kinase B (PKB), an important signal transduction node involved in cell survival under stress in both *D. discoideum* and mammalian cells. This peptide substrate reporter has been widely used to measure PKB activity in single cells.[8,10,11] Reporter loading was assessed based on the amount of peptide internalized by cells, the uniformity of loading between and within cells, the degradation rate of the reporter inside cells, and phosphorylation of the reporter in the presence and absence of a phosphatase inhibitor.

METHODS

Materials. HL-5 medium consisted of 14 g/L protease peptone no. 3, 7 g/L yeast extract, 11 mM monobasic potassium phosphate, and 3.5 mM dibasic sodium phosphate (pH 6.5). Development buffer (DB) was composed of 10 mM phosphate buffer (pH 6.5) with 1 mM $CaCl_2$ and 2 mM $MgCl_2$. SorCM buffer was composed of 14.5 mM monobasic potassium phosphate, 25 mM dibasic sodium phosphate, 10 mM $CaCl_2$ and 20 mM $MgCl_2$ (pH 6.5). H40 electroporation buffer consisted of 40 mM

HEPES (pH 7.0) and 1 mM $MgCl_2$. The run buffer used for capillary electrophoresis (CE) contained 100 mM borate and 15 mM sodium dodecyl sulfate (pH 11.4). The exogenous peptide used in this study was a fluorescently labeled substrate reporter for PKB, 6FAM-GRP-MeArg-AFTF-MeAla-NH_2.[10] The myristoylated form included an additional C-terminal cysteine residue that was linked by a disulfide bond to the peptide C-K(myr)-KKK-NH_2, where myr is a myristoyl group. Full-length peptides were synthesized and purified by Anaspec and peptide fragments were provided by the Allbritton laboratory at the University of North Carolina. All peptides were stored at -80 °C.

Cell culture. Axenic *D. discoideum* K-AX3 (strain DBS0236487) was obtained from the Dicty Stock Center and grown at room temperature with shaking at 180 rpm as described previously.[12] Cell densities were maintained between 5×10^3 and 5×10^6 cells/mL. Cell viability was determined by trypan blue staining.

Pinocytosis. For pinocytosis experiments, cells were resuspended at a density of 2×10^7 cells/mL in a hypertonic solution of 20% (w/v) PEG-1000 in DB with 0-150 µM peptide reporter. After incubating at room temperature, cells were washed and resuspended in DB for 10 min to lyse pinosomes. Cells were then pelleted and resuspended in 10% (w/v) PEG-1000 in DB or SorCM to prevent cell lysis due to prolonged osmotic stress.

Electroporation. For electroporation experiments, 2×10^6 cells were resuspended in 100 µL ice-cold H40 electroporation buffer containing 0-50 µM peptide reporter and transferred to an electroporation cuvette with a 2-mm gap. The electroporation system (GenePulser Xcell, BioRad) was configured for two 275-V, 4-ms square pulses with a 1-s pulse interval. After electroporation, the cells were washed and resuspended in SorCM buffer.

Myristoylation. For myristoylation experiments, cells were resuspended at a density of $2\text{-}2.5\times10^7$ cells/mL in CB containing 0-20 µM myristoylated peptide. After incubating, the cells were washed once and incubated for 1 min in 1 mM tris(2-carboxyethyl)phosphine (TCEP) in DB to cleave any myristoylated peptide remaining on the outer leaflet of the cell membranes. Cells were then washed and resuspended in DB or SorCM.

Fluorescence intensity measurements. For measurements of cell populations, a plate reader (SpectraMax M4) was used to measure the total fluorescence of 40-µL aliquots containing 2×10^6 cells per well in bottom-read 384-well plates (3542, Corning) with excitation at 485 nm, emission at 516 nm, and an emission cutoff of 515 nm. Imaging was performed on an Olympus IX73 epifluorescence microscope equipped with an LED illumination system (XCite 120), filter cube (FITC-3540C, Olympus), 60× objective (NA 0.9), and a CCD camera (1500M, Thor Labs). Data were collected using Micro-Manager (v.1.4.21) and analyzed in Image J.

Degradation and phosphorylation assays. Cells were loaded with peptide by each method as described above at external reporter concentrations of 35, 150, and 1 µM for electroporation, pinocytosis, and myristoylation experiments, respectively. For degradation assays, cells were washed and resuspended in DB or SorCM after loading and lysed at fixed time points by heating at 95 °C for 4 min. For phosphorylation assays, cells were resuspended in 10 mM phosphate buffer (pH 6.5) with 2 mM $MgSO_4$ plus 1 µM okadaic acid or ethanol (as a vehicle control) for 15 min prior to lysis by the addition of an equal volume of CE run buffer. Samples were injected by the application of pressure for 5 s, separated using an electric field of 393 V/cm in a 50 µm i.d. bare silica capillary with an effective length of 21 cm, and detected by laser-induced fluorescence at 488 nm (PA-800 Plus, Sciex). The detector was operated using the following default settings: medium dynamic range of 100 RFU, 4 Hz data acquisition rate, and normal filtering using 16-25 pts. Peaks in capillary electropherograms were identified by migration time and confirmed as needed by the addition of standards. Peak areas were determined using 32Karat (v. 10.1).

RESULTS AND DISCUSSION

Optimization of methods. Before comparing the three loading methods, we optimized each one for *D. discoideum* with respect to peptide loading and cell viability under nutrient-free buffer conditions. Under nutrient-poor conditions, *D. discoideum* undergoes a social life cycle during which cells aggregate and differentiate to form a fruiting body of 10,000-100,000 cells.[12] Because the biochemical basis for this phenomenon is an active area of research, we chose to use loading conditions that were compatible with social development.

Electroporation has been previously used to transfect *D. discoideum*,[5] and we tested established conditions from the literature and personal communications before selecting the parameters outlined in Table 1.[13–16] To ensure high cell viability after substrate loading, we chose an electroporation voltage that was lower than that used in previous works.

Table 1. Optimized conditions for each loading method.

Method	Buffer	Conditions	Viability (n = 6)
electroporation	H40	ice-cold two 4-ms, 275-V square pulses 1 s pulse interval	95 ± 4%
pinocytosis	20% (w/v) PEG-1000 in DB	room temp. 10-90 min	98 ± 1 %
myristoylation	DB	room temp. 1-20 min	100 ± 1%

To the best of our knowledge, pinocytic loading with osmotic lysis and myristoylation have not been previously used to load exogenous molecules into *D. discoideum*. Pinocytic loading with osmotic lysis of pinosomes to release material into the cytoplasm was first demonstrated in L929 mouse adipose tissue cells,[2] and commercial reagents are now marketed for use with mammalian cells.[17] Many cell types, including axenic *D. discoideum* strains,[18] spontaneously pinocytose the surrounding liquid medium. If the surrounding medium is made hypertonic relative to the normal cell medium, the resulting pinosomes may be lysed by an influx of water when the cells are resuspended in a hypotonic solution. The lysed pinosomes then release their contents into the cytoplasm.

Myristoylation is one example of a suite of loading methods based on covalent attachment of molecular cargo to a membrane-permeant species.[1] The proposed mechanism of loading is that the hydrophobic myristoyl chain inserts into the lipid

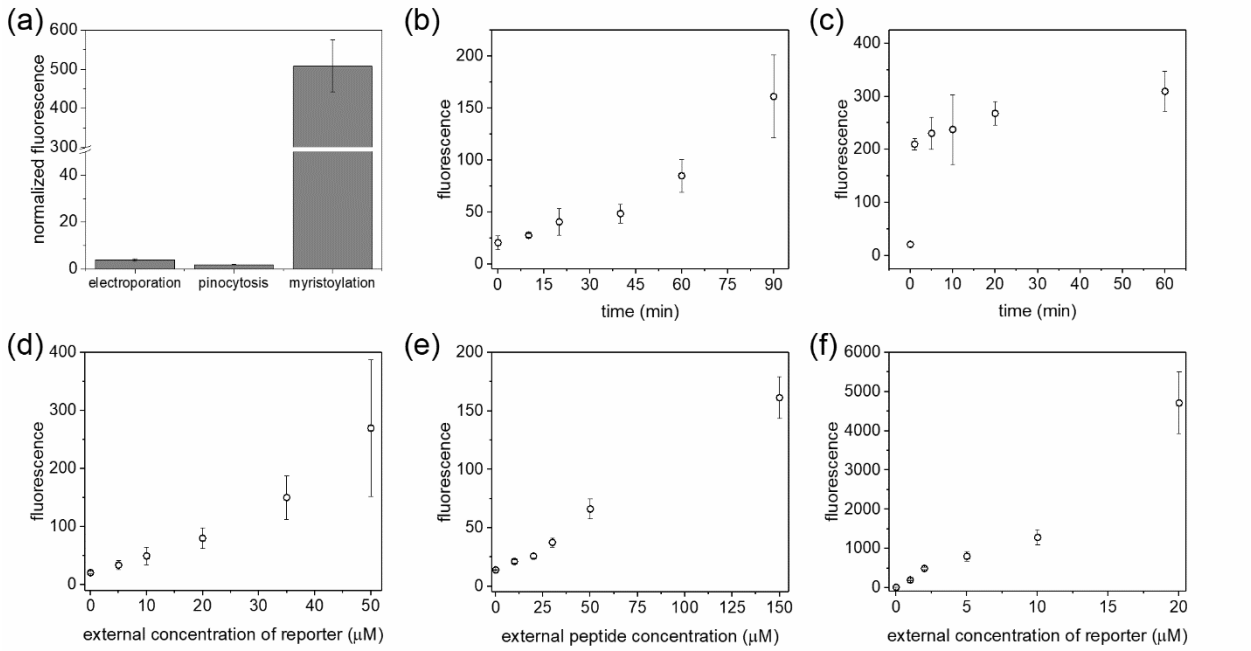

Figure 1. Ensemble fluorescence measurements of the amount of peptide substrate reporter loaded into *D. discoideum* cells by each method. (a) Normalized fluorescence intensity measurements of cells loaded with labeled peptide relative to unloaded control cells. For all samples, the external peptide concentration was 20 μM. Effect of incubation time on loading by (b) pinocytosis and (c) myristoylation. External peptide reporter concentrations were 50 and 1 μM, for pinocytosis and myristoylation, respectively. Effect of external peptide concentration on loading by (d) electroporation, (e) pinocytosis, and (f) myristoylation. Incubation times were 60 and 1 min, for pinocytosis and myristoylation, respectively. For all panels, $n = 3$ biological replicates were obtained on different days, and error bars indicate the standard deviations.

bilayer, carrying the cargo into the cell during a flip-flop to the inner membrane leaflet. When the myristoyl group is attached to the cargo by a disulfide bond, the cargo can be released in the reducing environment of the cytoplasm.[6] Although myristoylation-based loading has not been previously applied to *D. discoideum*, the cell membrane of this amoeba is similar to that of mammalian cells,[19] and we observed that loading conditions used for mammalian cells were readily transferred to this organism. The optimized methods produced detectable loading of the fluorescent peptide while maintaining cell viability at ≥ 95% (Table 1).

Loading efficiency. Of the three assayed methods, myristoylation was the most effective at transporting the peptide reporter across the outer membrane. Cells loaded using this technique were over 50-fold brighter than those loaded via electroporation or pinocytosis for the same external peptide concentration (Figure 1a). The cell membrane is a more favorable environment for the myristoyl chain than aqueous solution, which may drive loading of the peptide into cells. Reduction of the disulfide bond connecting the reporter to the myristoyl group may also help trap the reporter in the cell.[6] In contrast, electroporation-based loading is limited by the rate of diffusion of the peptide reporter and the duration of electropores (generally on the order of seconds-minutes).[20] Similarly, pinocytic loading rates are limited by the rate of cellular endocytosis. For pinocytosis, fluorescence increased roughly linearly with time, suggesting a constant rate of endocytosis of peptide-containing medium (Figure 1b). In contrast to pinocytic loading, loading of the myristoylated reporter occurred rapidly, and then slowed over time (Figure 1c). Similar results have been previously obtained in BA/F3 cells (a murine pro-B cell line).[6]

For all three methods, we were able to control the amount of peptide loaded (as measured by cell fluorescence) by varying the concentration of peptide in the loading medium (Figure 1d-f). The external reporter concentrations required to produce detectable loading varied between methods, but in all cases, the amount of peptide loaded into cells could be varied by at least one order of magnitude. Because the reporter concentration may affect measurements, researchers require the ability to tune the concentrations of loaded molecules to a particular assay. For example, peptide substrate reporters must be loaded at a sufficiently high concentration to be detectable and to compete with endogenous substrates; however, the concentration of reporter must also be low enough to avoid disrupting signaling pathways.

Loading uniformity. The average fluorescence values for ensemble measurements of each cell population are shown in Figure 1, but it is also useful to know how heterogeneous loading is between cells. Therefore, we used fluorescence microscopy to evaluate the uniformity of peptide loading. We quantified the average pixel intensity for 100 individual cells loaded by each method and constructed histograms of fluorescence intensity (Figure 2a-c). Because fluorescence values could not be less than zero, all three distributions were skewed compared to a Gaussian distribution; however, skewness values were more pronounced for electroporation and myristoylation (4.5 and 4.8, respectively) than for pinocytosis (2.5). These values reflect the fact that although most cells loaded by electroporation and myristoylation had similar fluorescence values, there were outlying tails in the distribution representing cells that were much brighter than the population median. In contrast, cell brightness was distributed more normally around the mean when cells were loaded by pinocytosis. The shape of the distribution is

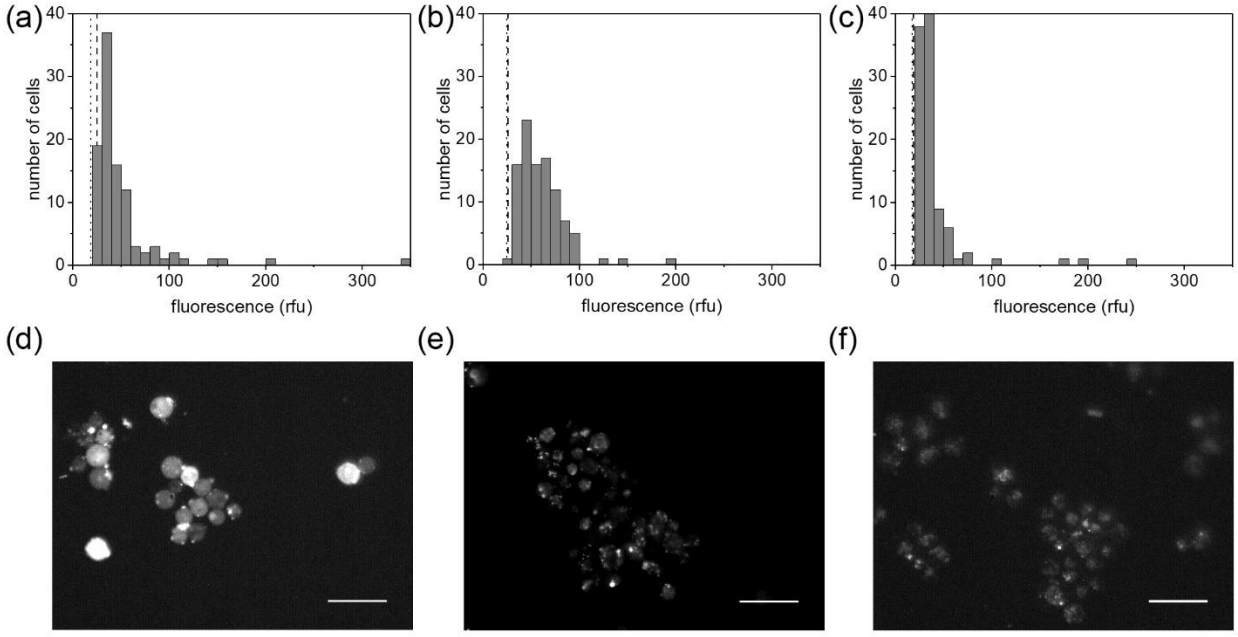

Figure 2. Evaluation of loading uniformity within and between cells for (a, d) electroporation, (b, e) pinocytosis, and (c, f) myristoylation. (a-c) Histograms showing fluorescence as determined by microscopy of n = 100 cells for each method. Dotted lines show the average background fluorescence of the images; dashed lines show the average fluorescence of unloaded control cells. (d-f) Fluorescence images of cells loaded by each method. The intensity and spatial scales are the same for all images (scale bar represents 25 μm). External concentrations of reporter were 35, 150, and 1 μM, for electroporation, pinocytosis, and myristoylation, respectively.

important because it determines how well the population average represents a "typical" cell. For pinocytosis, the mean fluorescence for the population reasonably reflected a typical (i.e., median) cell. In contrast, mean cell fluorescence was less representative of a typical cell loaded by electroporation or myristoylation. When ensemble measurements are made, this discrepancy may produce results that do not accurately reflect the behavior of a "typical" cell. For single cell experiments, variation in loading between cells has been exploited to examine the effects of concentration.[10,21,22] In this scenario, pinocytosis would produce data points that are more uniformly distributed by concentration.

We also evaluated the uniformity of reporter loading within individual cells, i.e., whether each method produced punctate loading, suggesting localization to membrane bound compartments, or uniform fluorescence, suggesting cytoplasmic loading (Figure 2d-f). While certain assays require localization of the reporter to specific compartments or organelles, in many instances uniform cytoplasmic loading is preferred. Electroporation resulted in fairly uniform fluorescence throughout the cytoplasm of individual cells (Figure 2d). Pinocytosis and myristoylation resulted in punctate fluorescence; some areas of cells (possibly endosomal compartments) appeared much brighter than the surrounding cytoplasm (Figure 2e, f). These qualitative observations were reflected in quantitative measurements of pixel intensity within cells. The standard deviation of pixel intensity within an individual cell was lower for cells loaded by electroporation (average value of 15 rfu) than for pinocytosis (22 rfu) or myristoylation (22 rfu). For pinocytic loading, localization was more pronounced if cells were not resuspended in hypotonic medium, suggesting that treatment with hypotonic medium was successful in lysing at least some of the pinosomes.

Effects on enzyme activity assays. We hypothesized that even when the concentration of peptide was held constant different loading methods would result in differences in enzymatic processing of the peptide reporter in cells. For example, localization of the peptide to lysosomal storage compartments could be associated with increased degradation of the reporter, while increased cell stress could activate PKB, resulting in phosphorylation of the reporter. To test this hypothesis, we loaded cells by each method, then lysed the cells and analyzed the resulting lysates by capillary electrophoresis with laser-induced fluorescence detection. Based on the data shown in Figure 1, we tailored the external concentration of the peptide to achieve similar average fluorescence levels for each method to minimize differences in enzymatic processing due to peptide concentration. In addition to providing information about peptide metabolism, the CE data provided an additional means of quantifying the amount of peptide loaded by each method. Based on calibration curves obtained on several days using purified peptide standards, we estimated the concentration of reporter loaded into cells by each method, which ranged from 0.1-0.9 μM. (This concentration range is an order-of-magnitude approximation, since the average cell volume was estimated from the literature[23] and because no internal standard was used to correct for variation in cell number, injection volume, or detector sensitivity.) These concentrations suggest uptake rates within an order of magnitude of previous measurements for electroporation (0.7-20% of the external concentration)[24] and pinocytosis (uptake of ~9 μm^3 min^{-1} $cell^{-1}$).[25–27] In general, uptake rates were lower than observed previously, perhaps because we optimized loading methods for high viability rather than for high loading efficiency.

We subsequently used the CE data to examine the rate of degradation and half-life of the reporter in cells loaded using each method. Intracellular peptidases rapidly degrade most peptides

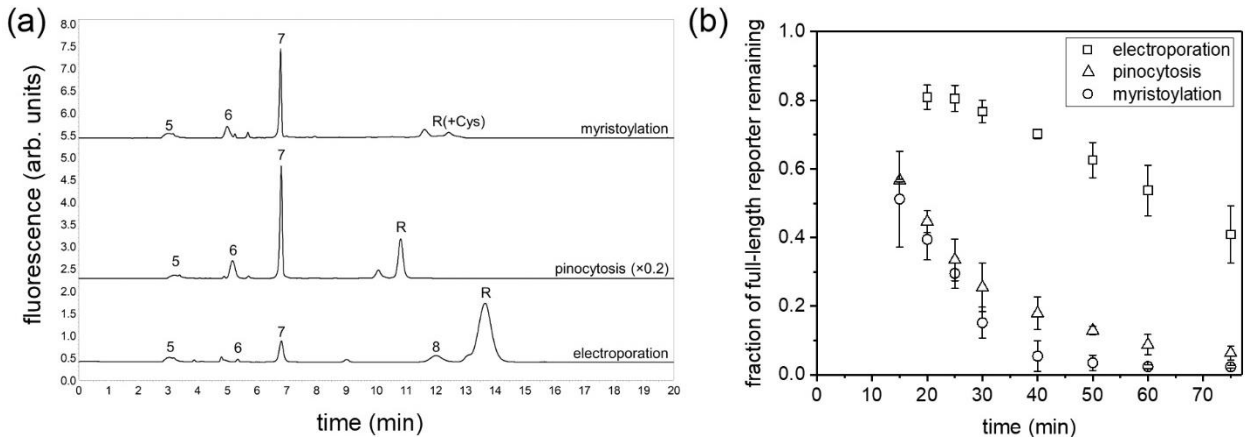

Figure 3. (a) Representative capillary electropherograms showing degradation of the peptide substrate reporter by peptidase activity in intact cells 30 min after loading by each method. Peak labels indicate the number of amino acid residues remaining on the N-terminally labeled fragments of the reporter; R indicates the full-length, nine amino acid reporter and R+Cys indicates the full-length reporter plus a C-terminal cysteine residue for the myristoylated peptide. All electropherograms are aligned to the 7-amino acid fragment peak. (b) Degradation of the full-length reporter over time by loading method. Error bars show the standard deviation for $n = 3$ biological replicates.

into amino acids such that they can be recycled into new proteins.[28] The reporter studied in this work was previously optimized for stability in HeLa lysates, and our previous work demonstrated a comparable half-life in *D. discoideum* lysates.[29] Knowing the half-life of the intact reporter is important because it provides an estimate of the time scale upon which phosphorylation assays must be completed to minimize interference from reporter degradation. The electropherograms for samples from cells loaded using each method were qualitatively similar with respect to the peptide fragments detected (Figure 3a) and resembled previously observed fragmentation patterns in *D. discoideum* lysates. This similarity suggested that degradation likely proceeded via similar pathways in both intact cells and lysates. In a previous study of lysates, the highest rate constants corresponded to the direct production of the 6- and 7-amino acid fragments from the full-length reporter and the production of the 6-amino acid fragment from the 8-amino acid fragment.[29] However, the reporter degradation rate differed markedly between methods (Figure 3b). The peptide was degraded most rapidly after loading by myristoylation (half-life = 12 ± 1 min), while pinocytic loading produced slightly slower degradation (half-life = 19 ± 2 min). Importantly, for the latter method it appears that degradation did not begin until the cells were resuspended in hypotonic media, since > 50% of the peptide remained intact at the first time point, 15 min after resuspension in hypotonic media and over an hour after loading was initiated. This result suggests that the peptide remained sequestered in the pinosomes at least until the cells were exposed to the hypotonic media. Electroporation produced the slowest degradation rate among the three methods (half-life = 53 ± 15 min). This half-life was still shorter than was observed previously in lysates (82-103 min),[29] likely because of higher protein concentrations in the intact cells.

The peptide substrate reporter used in these studies was designed to report on the phosphorylation activity of PKB in human cells. Peptide substrate reporters complement established techniques to measure cell signaling, such as immunoblotting, because they provide a direct readout of signaling activity and are compatible with single-cell measurements. Although this reporter has not been used previously to measure PKB activity in *D. discoideum*, a homolog of this kinase is present in this organism and is transiently activated during chemotactic signaling.[30] We studied the effect of okadaic acid on phosphorylation of the reporter in cells loaded by each method. Okadaic acid is a naturally occurring toxin that inhibits protein phosphatases, including PP2A.[31,32] In both human and *D. discoideum* cells, PP2A is a negative regulator of PKB activity,[33,34] so treatment with okadaic acid was expected to increase baseline phosphorylation activity of the peptide substrate reporter. Although the absolute levels of phosphorylation (~1%) were lower than those that have been observed previously in human cells, previous work has focused on cell types with pathological PKB activity, such as cancer cell lines and patient tissue.[8,10,11] Additionally, the peptide substrate reporter used in this study was designed for use with the human enzyme, and its amino acid sequence may not be optimal for the *D. discoideum* PKB homolog. Indeed, our on-going work in cell lysates suggests that phosphorylation of the reporter reflects known physiological trends in PKB activity in *D. discoideum* but that phosphorylation rates are low compared to other substrates (unpublished data). Despite the low levels of observed phosphorylation, the results showed clear differences between loading methods. In fact, phosphorylation activity toward the peptide was only observed in cells loaded by electroporation, as cells loaded by pinocytosis and myristoylation lacked a peak for the phosphorylated reporter (Figure 4). In cells loaded by electroporation, okadaic acid treatment resulted in a reproducible 1.42 ± 0.05 fold increase in phosphorylation activity compared to vehicle controls ($n = 3$ biological replicates).

The observed differences in enzymatic processing of the reporter between loading methods can likely be explained by differences in the localization of the reporter. Localization of the peptide to lysosomal compartments would enhance its degradation and reduce phosphorylation, as was observed in cells loaded by pinocytosis and by myristoylation. Since both pinocytosis with osmotic lysis and myristoylation have been used to produce cytoplasmic loading in other cells types,[2,6] these data highlight the need to confirm and characterize loading when adapting an established method to a new cell type.

CONCLUSIONS

In summary, we demonstrated that loading method influences the fate of exogenous molecules in cells through systematic

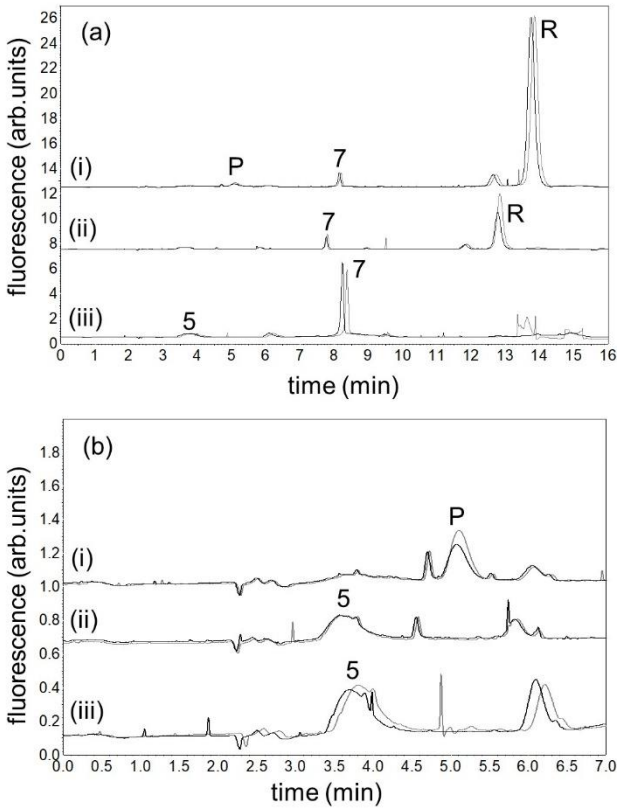

Figure 4. (a) Sample electropherograms of lysates from cells loaded by (i) electroporation, (ii) pinocytosis, or (iii) myristoylation followed by treatment with 1 μM okadaic acid (gray) or vehicle control (black). (b) Enlarged view of the electropherograms from 0 to 7 min. Peak labels indicate the number of amino acid residues remaining on the N-terminally labeled fragments of the reporter; R indicates the full-length reporter, and P indicates the phosphorylated reporter. The presence or absence of phosphorylated reporter was confirmed by addition of a standard.

study of three loading methods in *D. discoideum*. *D. discoideum* is an important model organism in studies of cell migration and chemotaxis, but there is limited information describing how to load exogenous molecules into these cells. We have optimized three different loading methods, providing a range of protocols for future work. Each method has been characterized with respect to the amount of peptide loaded, the timescale of loading, heterogeneity between individual cells, and the localization and metabolism of the loaded molecule. For peptide substrate reporters, such as the one studied here, this information is critical for researchers to make informed choices of loading methods. While the specific conditions and characteristics of the loading methods used in this study may not be directly transferrable to studies in human or other cell types, the more general finding that the choice of loading method influences the stability and biochemical fate of exogenous molecules in cells is transferrable. These data are a reminder that in complex systems, such as living cells, opportunities for artifacts arise at each step of an experiment.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡These authors contributed equally.

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