Real-Time Quantification of Cell Internalization Kinetics by Functionalized Bioluminescent Nanoprobes

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A cell is the basic functional and biological unit of living organisms. Cell membranes serve as a physical barrier separating the intracellular endosomal compartments from the extracellular space. Substances, including nutrients, are translocated across the cell membranes through endocytosis and exocytosis, maintaining cellular metabolism and systemic homeostasis. Beyond such processes, there has been great interest to deliver biomolecules, such as proteins and genes, to cells for gene editing,[1,2] cell reprogramming,[3,4] therapy,[5–7] and other purposes.[8] Quantifying translocation kinetics is of fundamental and clinical importance, which may provide quantitative measures that reflect cellular metabolic activity, as well as essential parameters toward the rational design of delivery vectors.

To quantify the internalization process, fluorescence microscopy[9,10] and fluorescence-activated cell sorting (FACS) techniques[11,12] are frequently used tools. However, the fluorescence microscopy presents the semi-quantitative kinetics information in cellular level rather
than statistical quantification. And FACS, on the other hand, requires pre-treatment steps (e.g., rinse, fixation, or detachment of cells) and provide only an incomplete understanding of an internalization process at specific time points. It is also difficult to distinguish the fluorescence signals from fluorophores that are located within the cells or on the cell membranes.\textsuperscript{23} In addition, it is challenging to quantify the roles of the surface characteristics (e.g., charge and functional moieties) on the delivery outcomes. Inorganic nanoparticles (e.g., gold\textsuperscript{14} and iron oxide\textsuperscript{15}) were also employed as model vectors, in which the amounts of the particles internalized were quantified by atomic absorption spectroscopy,\textsuperscript{16,17} electron microscopy,\textsuperscript{18} or magnetophoresis.\textsuperscript{19} Nevertheless, these methods can only obtain information about the internalization process in discrete time.

We report herein a real-time cell internalization kinetics assay (RCIKA) using bioluminescent nanoparticles as the probes. Bioluminescence commonly exists in living organisms; one example is firefly luciferase (FL), which catalyzes the oxidation of luciferin in the presence of adenosine-5’-triphosphate (ATP) with light emission. ATP is a common phosphate donor with a concentration of 1–10 × 10^{-3} \text{M}\textsuperscript{20,21} in cytosol or 10^{-3}–1 × 10^{-6} \text{M} in extracellular environments.\textsuperscript{22,23} The substantially higher ATP concentration in cytosols makes FL nanoparticles effective probes for cell internalization, during which a sudden increase of ATP concentration enables the bioluminescent reaction. The real-time bioluminescent intensity is converted to real-time concentration of the nanoparticles internalized, enabling quantitative measurement of cell-internalization kinetics with high sensitivity. Furthermore, the essential surface characteristics of these bioluminescent nanoparticles, such as surface charge and functional moieties (e.g., cell penetrative moieties, targeting moieties, and PEG moieties), can be tailored to constitute a tool for vector design.

Bioluminescent nanoparticles were synthesized using in situ polymerization.\textsuperscript{24,25} Briefly, FL molecules were conjugated with polymerizable acryl groups and added to an aqueous solution containing acrylamide (AAM, neutrally-charged monomer), N-(3-aminopropyl) methacrylamide (APM, positively-charged monomer), and N, N’-methylenebisacrylamide (BIS, crosslinker). Driven by noncovalent interactions, the monomers and the crosslinker were enriched around the FL molecules. Subsequent polymerization formed a thin layer of the polymer, encapsulating individual FL molecules, forming FL nanocapsules denoted as nFL. Figure 1Ai shows the zeta potential and size of a series of nFL synthesized with various APM ratios APM \text{AAM} \approx 1:5600. The zeta potential of the nanocapsules increases from 0 to 15 mV linearly with the APM ratio. Figure 1Aiv further compares the zeta potential of native FL and a representative nFL, which increases from \approx -7.9 to 6.9 mV. The increased zeta potential indicates the growth of a cationic shell around the FL, which prevents the decline of FL enzyme activity by inhibiting the protease degradation (Figure S1, Supporting Information), while remaining low cytotoxicity (Figure S2) and allowing effective transport of luciferin and ATP (Figure S3, Supporting Information).

These nFL exhibit a similar size (\approx 26 nm in diameter, Figure 1Ai). Figure 1Aii shows a representative transmission electron microscopy (TEM) image of nFL with a zeta potential of 6 mV (denoted as nFL(6 mV)), similar notations will be used hereinafter for nFL, revealing a spherical morphology with an average diameter of 25 nm. Figure 1Aiii further exhibits the size distribution of native FL and nFL(6 mV) by dynamic light scattering (DLS), indicating an average diameter of 8 and 25 nm, respectively. Our previous work has demonstrated that nanocapsules synthesized using this method exhibit a single-protein structure, in which each nanocapsule contains a single-protein core and a thin polymer shell.\textsuperscript{24,25} It is worth noting that nanoparticles with a diameter in the range of 20–30 nm require the least energy to deform the cell membranes during internalization, enabling the most effective cell penetration.\textsuperscript{26}

Besides the uniform size and tunable surface charge, functional moieties were conjugated to nFL to tailor the surface characteristics. For example, PEG (molecular weight 2000, PEG2000) was conjugated to nFL(9 mV) at a molar ratio of 12:1 and 23:1. The PEGylated nFL, denoted as PEG1-nFL and PEG2-nFL, respectively, were labeled with fluorescein isothiocyanate (FITC) and incubated with 4T1 cells. The cells exposed to the PEGylated nFL exhibit significantly reduced fluorescence intensity (reduced endocytosis) in comparison with nFL(9 mV) (Figure 1Bi). FACS confirms that the internalization of PEG1-nFL and PEG2-nFL decreases \approx 30% and 54%, respectively (Figure 1Bii). The targeting peptide, arginine–glycine–aspartic acid (RGD), was conjugated to nFL(9 mV) (denoted as RGD-nFL, \approx 2.5 RGD per nFL). Figure 1Ci,ii presents the fluorescence images and FACS of 4T1, A549, and HeLa cells incubated with RGD-nFL. The A549 and HeLa cells exhibit a 1.5-fold and 1.2-fold increase of cellular uptake in comparison with the 4T1 cells due to their overexpression of the RGD receptors (integrin \alpha\beta_1).\textsuperscript{27–29} Similarly, TAT, a cell penetrative peptide, was conjugated to nFL(9 mV) (denoted as TAT-nFL, \approx 2.3 TAT per nFL). Figure 1Di,ii shows the fluorescence microscopy images and FACS of 4T1 cells incubated with nFL(9 mV) and TAT-nFL, respectively, indicating a 2.6-fold increase of uptake for TAT-nFL.

The ability to synthesize nFL with uniform size, tunable surface charge, and functionality provides a novel class of bioluminescent probes for real-time monitoring the kinetics of cell internalization. As depicted in Figure 2A, upon internalization of nFL by cells pre-incubated with luciferin, the internalized nFL catalyzes the oxidation of luciferin emitting bioluminescence, of which the intensity can be quantified by

\[
\text{RLU}_i = \frac{K_{\text{cat}}[S]}{K_{\text{cat}} + [S]} \cdot nFL \cdot e^{-kt} \cdot A \cdot N
\]

where RLU\textsubscript{i} is the relative light units per second (RLU s^{-1}); t is time; N is the number of cells in a well of a 96-well plate; and A is a conversion factor between RLU per mole of luciferin reacted, which is 5.73 \times 10^{3} RLU mol^{-1}\textsuperscript{30} (Section S3.13.4, Supporting Information). The term \frac{K_{\text{cat}}[S]}{K_{\text{cat}} + [S]} represents the rate of the reaction of luciferin, where K_{\text{cat}} and K_{\text{in}} are the turnover number and Michaelis constant of nFL measured by the Lineweaver–Burk plot; and [S] and [nFL] are the concentration of luciferin and nFL in the cytosol, respectively. As shown in Section S3.13.2 (Supporting Information), the
amount of luciferin consumed in this process is negligible, and \( [S] \) is approximately equal to the initial luciferin concentration in cytosol \((2.0 \times 10^{-3} \text{ M})\). In addition, compared with the ATP concentration within a cell \((1-10 \times 10^{-3} \text{ M})\) \(^{20,21}\), the concentration of the nanocapsules delivered to the cell is several orders in lower magnitude, ensuring the accuracy of this kinetics term. The term \(e^{-dkt} \) is used to compensate for the decay of nFL activity, where \(k_d\) is the decay constant determined by the Hough transform (Figure S5, Supporting Information). Detailed information is provided in Section S3.13 (Supporting Information).

One essential criterion for RCIKA is that the emission of bioluminescence occurs only after nFL is internalized into the cells where ATP presents. To confirm this criterion, FITC-labeled nFL(0 mM) and FITC-labeled nFL(6 mM) were incubated with 4T1 cells. As expected, fluorescence images suggest that nFL(6 mM) with a positive charge is internalized effectively, whereas nFL(0 mM) with a neutral charge exhibits negligible uptake (Figure 2B). As shown in Figure 2C, nFL(6 mM) or nFL(0 mM) in the absence of ATP shows a background intensity of \(\approx 50\) RLU. In contrast, 4T1 cells incubated with nFL(6 mM) emit intensive bioluminescence of 6174 RLU, in comparison with 52 RLU for the cells nFL(0 mM). Adding \(10 \times 10^{-3}\) M of ATP to the media further intensifies the intensity to 7050 and 2599 RLU, respectively, confirming that bioluminescence only occurs after internalization and the extracellular signals are negligible.

Figure 2D presents a representative RLU profile of 4T1 cells exposed to \(4.6 \times 10^{-6}\) M nFL(6 mM). The light intensity increases with time, reaches peak intensity at \(\approx 10\) min,
and decreases exponentially afterward, consistent with an increasing uptake of the nFL with decaying activity. Figure 2E shows the temporal concentration of nFL within the cells, denoted as $[\text{nFL}_{\text{in}}]$, based on $k_d$, $K_m$, and $K_{\text{cat}}$ of $1.08 \times 10^{-3}$ s$^{-1}$, $0.31 \times 10^{-3}$ m, and $0.022$ s$^{-1}$, respectively (Section S3.13, Supporting Information). As expected, $[\text{nFL}_{\text{in}}]$ increases with time and reaches a plateau concentration, $[\text{nFL}_{\text{plateau}}]$, of $\approx 0.49 \times 10^{-9}$ m at $\approx 47.4$ min. $[\text{nFL}_{\text{in}}]$ increases linearly at the initial stage (e.g., within 10 min) with a slope of 10.35 pm min$^{-1}$, which is defined hereafter as the initial rate of uptake (IRU), an important parameter reflecting the rate of uptake of cells. We expect that engineered luciferase with improved stability and sensitivity can be used to synthesize bioluminescent probes, which should extend the time scale of monitoring and lead to improved assay accuracy.

The reliability of RCIKA has been validated by FACS, in which FITC-labeled nFL(6 mV) and nBSA(6 mV) (nanocapsules of bovine serum albumin) were incubated with 4T1 cells, and fluorescence intensities of the cells were examined at different time points (Figure S4A,B, Supporting Information). The green and red cubes, respectively, show the relative fluorescence intensities of 4T1 cells incubated with FITC-labeled nFL(6 mV) or FITC-labeled nBSA(6 mV) for different times, respectively. The fluorescence intensities were measured by FACS and normalized by their plateau intensity.

RCIKA provides a real-time tool for fast quantification of cell internalization processes. For example, previous investigations report that the positive surface charge of nanoparticles facilitates cell internalization.[31] Such effects can be quantified using nFL with different surface charge, nFL(3 mV), nFL(6 mV), and nFL(9 mV). Figure 3A shows the temporal $[\text{nFL}_{\text{in}}]$ profiles of 4T1 cells incubated with $6.56 \times 10^{-6}$ m of these nFL, which exhibit an IRU of 15.96, 18.69, and 45.42 pm min$^{-1}$, respectively. This result indicates that a higher surface charge indeed results in faster and more uptake. To further quantify the uptake kinetics, IRU of 4T1 cells at different extracellular concentrations of nFL, denoted as $[\text{nFL}_{\text{out}}]$, were measured. It was found that the uptake follows a second-order kinetics, $\text{IRU} = a[\text{nFL}_{\text{out}}]^2$, where $a$ is a rate constant increasing from 0.33, 0.46, to 1.06 M$^{-1}$ min$^{-1}$, respectively (Figure 3B). Figure 3C further plots the time required to reach half of the plateau concentration (HPCT) at different $[\text{nFL}_{\text{out}}]$, suggesting a linear increase with a rate of 0.76, 1.37, and 1.34 min $\mu$m$^{-1}$, respectively. The prolonged HPCT and increased IRU with increasing surface charge result in higher $[\text{nFL}_{\text{plateau}}]$. 

Figure 2. Quantification of cell internalization kinetics. A) A schematic of the internalization of nFL that catalyzes the bioluminescent reaction of luciferin in the presence of ATP. B) Fluorescence images of 4T1 cell after incubation with $4.6 \times 10^{-6}$ M FITC-labeled nFL (6 mV) or nFL (0 mV) for 1 h. The nuclei were stained with DAPI (blue color). C) RLU of nFL(6 mV) and nFL(0 mV) and of 4T1 cells exposed to nFL(6 mV) or nFL(0 mV) with and without the addition of ATP. Data represent mean ± s.d. from three independent experiments. D) Temporal RLU profile of 4T1 cells exposed to $4.6 \times 10^{-6}$ m nFL(6 mV). E) Temporal $[\text{nFL}_{\text{in}}]$ profile of nFL(6 mV) being internalized to 4T1 cells calculated from the RLU profile in D). The green cubes and red cubes are the relative fluorescence intensities of 4T1 cells incubated with FITC-labeled nFL(6 mV) or FITC-labeled nBSA(6 mV) for different times, respectively. The fluorescence intensities were measured by FACS and normalized by their plateau intensity.
Figure 3. Cell internalization kinetics of nFL with varied surface charge. A) Temporal [nFL\textsubscript{in}] profiles of 4T1 cells exposed to 6.56 \times 10^{-6} \text{ M} of nFL(3 mV), nFL(6 mV), and nFL(9 mV), respectively. B) Initial rate of uptake (IRU), C) the time required to reach half of the plateau concentration (HPCT), D) plateau concentration [nFL\textsubscript{plateau}], and E) partition coefficient (K\textsubscript{eq}) of 4T1 cells exposed to nFL(3 mV), nFL(6 mV), and nFL(9 mV) with a series of initial concentrations ranging from 0.66 to 6.56 \times 10^{-6} \text{ M}. Data represent mean \pm s.e.m. from three independent experiments for (B–E).

Figure 3D further presents [nFL\textsubscript{plateau}] at different [nFL\textsubscript{out}], revealing a second-order correlation of [nFL\textsubscript{plateau}] = c[nFL\textsubscript{out}]\textsuperscript{2}, where c is a constant of 11.2, 19.8, and 57.7 m\textsuperscript{-1}, respectively, increasing with an increase of surface charge. Under this circumstance, the rate of endocytosis and exocytosis can be written as \(k\textsubscript{en}[nFL\textsubscript{out}]^2\) and \(k\textsubscript{ex}[nFL\textsubscript{plateau}]\), where \(k\textsubscript{en}\) and \(k\textsubscript{ex}\) are the apparent rate constant at equilibrium, respectively (details can be found in Section S3.14, Supporting Information). Since the rate of endocytosis equals to the rate of exocytosis, it is found that \(c\) equals to \(k\textsubscript{en}/k\textsubscript{ex}\), reflecting the accumulation tendency of nanoparticles in the cells. This uptake equation can be rewritten as \([nFL\textsubscript{plateau}] = k\textsubscript{en}/k\textsubscript{ex} [nFL\textsubscript{out}]\), suggesting that the final uptake amount is determined by the rate constants of endocytosis and exocytosis, as well as the extracellular concentration of the nanoparticles. Such findings provide essential dosage guidance for intracellular delivery and therapy with balanced efficacy and toxicity.

Assuming that \(k\textsubscript{en}\) remains unchanged during the internalization process, and \(k\textsubscript{en}\) equals to the rate constant of IRU, then \(k\textsubscript{ex}\) can be estimated. Consistently, \(k\textsubscript{ex}\) increases (0.33, 0.46, and 1.06 m\textsuperscript{-1} min\textsuperscript{-1}) while \(k\textsubscript{ex}\) decreases (2.9 \times 10\textsuperscript{-2}, 2.2 \times 10\textsuperscript{-2}, and 1.8 \times 10\textsuperscript{-2} min\textsuperscript{-1}) with increasing surface charge for nFL(3 mV), nFL(6 mV), and nFL(9 mV), respectively (Figure S6, Supporting Information). This observation indicates that cells exhibit faster endocytosis and slower exocytosis for nanoparticles with a higher surface charge, which is probably due to the increased electrostatic interactions with the receptors involved.

Partition coefficients of therapeutic agents between the intracellular compartments and extracellular environment is another essential parameter. Figure 3E plots the partition coefficients of the nFL, estimated by \(K\textsubscript{eq} = [nFL\textsubscript{plateau}]/[nFL\textsubscript{out}]\). It was found a higher surface charge results in a higher \(K\textsubscript{eq}\) and that \(K\textsubscript{eq}\) increases linearly versus [nFL\textsubscript{out}] with a slope equal to \(k\textsubscript{en}/k\textsubscript{ex}\). For a nonliving system, the partition coefficient of a substance between two phases is generally independent of concentration. The observed concentration dependency may be attributed to the bioenergy input during the internalization, which is a topic that requires further investigation.

As mentioned above, extensive efforts have been made to functionalize nanoparticulate vectors for improved outcomes. RCIKA also constitutes a tool to quantify the efficacy of these functional moieties. For example, PEGylation reduces toxicity and immunogenicity, and prolongs the circulation time of nanoparticles, whereas it also reduces cell penetrative ability. To quantify this effect, 4T1 cells were exposed to nFL(9 mV), PEG1-nFL (12 chains per nFL), and PEG2-nFL (23 chains per nFL) with decreasing IRU of 23.9, 19.4, and 5.0 pm\textsuperscript{-1} and [nFL\textsubscript{plateau}] of 1.3, 0.8, and 0.15 \times 10\textsuperscript{-3} M, respectively (Figure 4A). Increasing the PEG chain density rapidly reduces both the uptake rate and amount. Similarly, targeting peptides are commonly used to provide targeting capability. To quantify the targeting efficacy, RGD and RAD (arginine–alanine–aspartic acid) were conjugated to nFL(9 mV) (=2.5 RGD or RAD peptides per nFL) and delivered to 4T1, HeLa, and A549 (Figure 4B,C,D, respectively). An insignificant targeting effect (similar to IRU and [nFL\textsubscript{plateau}] was observed in 4T1 cells, which is consistent with their lack of RGD receptors.\cite{29} HeLa cells and A549 cells exhibited an increasing difference in IRU and [nFL\textsubscript{plateau}], consistent with their increasing number of RGD receptors.\cite{67,68} The targeting ability can be quantified by the ratio of [nFL\textsubscript{plateau}] or IRU, which is 0.95, 1.17, and 1.33 based on [nFL\textsubscript{plateau}] or 0.94, 1.66, and 1.96 based on IRU for 4T1, HeLa, and A549 cells, respectively. In this context, RCIKA offers a tool to quantify the targeting ability of various targeting moieties, providing an effective tool for drug development.

Beyond the ability to quantify targeting capability, RCIKA also provides a tool for the rapid screening of functional moieties for...
effective cell penetration. For a demonstration, a pool of CPPs R10, TAT, Antp, pVEC, NrTP, 14–21, TCTP#35, and PreS2, which contain varied sequences and charge residues, were conjugated to nFL(9 mV) (≈2.3 peptides per nFL) and delivered to 4T1, A549, and HeLa cells without compromising the cell viability or the level of intracellular ATP (Table S1, Figures S7 and S8, Supporting Information). Figure 4E,F shows the heat maps of [nFL plateau] and IRU. From left to right, 4T1 cells exhibit decreasing [nFL plateau] and IRU, which is consistent with previous results.[32,33] The cell penetrative ability (CPA) can be quantified by comparing [nFL plateau] or IRU versus the controls (nFL(9 mV) or non-CPP). For example, R10, TAT, Antp, and pVEC show a CPA of 25, 20, 21, and 19 based on [nFL plateau] or 14, 12, 12, and 10 based on IRU, respectively. Interestingly, CPA is cell-type dependent, and A549 and HeLa cells show significantly lower CPA in comparison with 4T1 cells. Considering the mature technologies of synthesizing peptides with designed sequences, RCIKA enables the rapid screening of peptides for various applications.

Using luciferase nanocapsules as the probes, RCIKA provides a real-time cell internationalization kinetics assay, yielding important kinetics parameters, such as the rate of uptake, half of the plateau concentration time, plateau concentration, endocytosis rate constant, exocytosis rate constant, and partition coefficient. The efficacy of functional moieties on intracellular delivery, such as targeting ability and cell penetrative ability, can also be quantified. Such kinetic parameters are essential to enable more effective intracellular delivery with better outcomes. Combining peptide synthesis techniques, RCIKA will also serve as a fast-screening tool for drug development and other applications. In a broader scope of cell biology and oncology, RCIKA can provide a quantitative measure for cellular metabolism, a hallmark of tumorigenesis, which is essential for oncogenic research, drug development, cancer therapy, and other applications.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

D.W. and Y.Y. contributed equally to this work. This work was supported by the ENN Center for Nanomedicine and Energy Conversion. The authors would like to thank the California NanoSystem Institute for technical assistance and Jun Ma for suggestions of the mathematical quantification. 4T1, HeLa, A549, and J774A.1 cells were purchased from American Type Culture Collection (ATCC).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

bioluminescence, cell internalization kinetics, firefly luciferase, nanocapsules, real-time quantification

Received: April 18, 2019
Revised: June 14, 2019
Published online:


