Chitosan-assisted permeabilization of ATP–biotin for live cell kinase-catalyzed biotinylation

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Kinases are essential cell signaling enzymes that phosphorylate protein substrates using ATP as the universal cosubstrate. A wide variety of ATP analogs have been used in kinase research, although the studies are limited by the cell impermeability of ATP. Here we describe the use of the cationic polymer deacetylated chitosan to permeabilize ATP analogs for live cell applications, including kinase-catalyzed biotinylation.

Cell signaling pathways regulate a variety of metabolic processes in living cells. Protein kinases mediate signaling by catalyzing phosphorylation of proteins using ATP as a cosubstrate (Figure 1A) [1]. Alterations in the function of protein kinases can cause cancer [2], Parkinson's [3,4], diabetes mellitus [5] and cardiovascular diseases. With over 500 kinases and thousands of substrates, chemical methods have been essential in the study of kinase-related diseases [6].

One powerful approach to study kinase activity utilizes ATP analogs [7] that contain sugar [8], base [9–11] or triphosphate modifications [12–21]. As one example, our laboratory and others have

used γ -phosphate-modified ATP analogs, including ATP–biotin (Figure 1B), as cosubstrates for kinase-catalyzed labeling reactions [22–27]. ATP–biotin was used to transfer a phosphoramidylbiotin group to proteins through kinase-catalyzed labeling (Figure 1A) [22,28]. The biotin group has the advantage of binding to the streptavidin protein, which allows purification and visualization of biotin-labeled phosphoproteins [29]. Unfortunately, kinase-catalyzed labeling is limited to *in vitro* lysate-based studies due to the cell impermeability of ATP analogs. The impermeability of ATP analogs has challenged the study of kinase activity and cell signaling in a cellular context. Cell-based experiments would maintain the

natural compartmentalization of the cell, which would make kinase studies with ATP analogs more physiologically relevant.

Recently, we reported the first intrinsically cell permeable ATP analog, ATP–polyamine–biotin (APB, Figure 1B), which is compatible with live-cell kinase-catalyzed labeling [27]. The cell permeability of APB is due to the presence of a polyamine linker, which is positively charged under physiological conditions and partially neutralizes the negative charges of the triphosphate. Several additives have also been used to permeabilize ATP and other small molecules. Of these methods, the mild non-ionic detergent digitonin has been used by several research groups to

METHOD SUMMARY

ATP and its analogs are intrinsically cell impermeable, making cell-based studies with ATP analogs impractical. Here, we document that deacetylated chitosan promoted the delivery of ATP analogs into cells. As an application of the method, cells treated with an ATP–biotin analog/chitosan mixture demonstrated kinase-catalyzed biotinylation of proteins in live cells.

Figure 1. Schemes representing kinase-catalyzed phosphorylation, ATP analogs and deacetylase chitosan. (A) Kinase-catalyzed phosphorylation of proteins using ATP or the ATP analogs, ATP-biotin or APB. (B) The structures of ATP ($X = O$), ATP-biotin, and APB. (C) The structure of deacetylase chitosan. APB: ATP-polyamine-biotin.

permeabilize ATP analogs [30]. Digitonin interacts with cholesterol and β -hydroxy glycerol on membranes to facilitate permeabilization [31]. Unfortunately, most digitonin-assisted permeabilization studies have been performed with cells in buffer instead of live growth conditions in media [20,32]. The physiological relevance of studies using digitonin-permeabilized cells might be compromised due to these ‘pseudo *in cellulo*’ conditions. In addition, exogenously added ATP partially reversed permeabilization by digitonin, which might lead to low efficiency [32]. Alternative additives that maintain normal growth conditions and high permeabilization efficiency would have immediate use to analyze kinase-mediated

signaling pathways with ATP analogs in live cells.

Deacetylated chitosan (DC, Figure 1C) was reported to chelate ATP and nucleotide-containing drugs to form cell permeable nanoparticles [33]. The cell permeability depended on the ratio of positively charged nitrogens in DC to negatively charged phosphates in ATP (N:P ratio), with the best ratio being 1:1 [33]. In addition, DC was used under normal cell growth conditions in media, allowing experiments to be performed using physiologically relevant conditions. Here, we report the first application of DC as a vehicle to deliver ATP analogs into cells for *in cellulo* kinase-catalyzed labeling. We also compared the

permeabilization and labeling efficiency of DC to digitonin and found higher efficiency with DC. DC permeabilization offers a powerful alternative to use of physiologically compromised digitonin permeabilization or tedious synthesis of intrinsically permeable ATP analogs.

Materials & methods

Fluorescence microscopy assay

HeLa cells (160,000) were grown on a glass cover slip in a 12-well plate overnight in F-12 growth media (500 μ l, F-12 media, 10% FBS, penicillin [9 units], and streptomycin [9 units]) at 37°C in a 5% CO_2 environment [34]. Cells were then incubated in serum-free F12 media for 1 h at 37°C in a 5% CO_2 environment. After the media was removed, cells were incubated for 2 h at 37°C in a 5% CO_2 environment with serum free media (in case of DC) or P buffer (in case of digitonin) (400 μ l) containing one of the following: ATP (2 mM), ATP-biotin alone (2 mM), DC alone (1.9 mg/ml), digitonin alone (20 μ g/ml), ATP-biotin/DC (2 mM/1.9 mg/ml), or ATP-biotin/digitonin (2 mM/20 μ g/ml). Cells were washed with DPBS (500 μ l, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4, 137.9 mM NaCl, 2.6 mM KCl) three times, followed by washing with PBS (500 μ l, 2 mM KH_2PO_4 , 10 mM Na_2HPO_4 , pH 7.4, 137 mM NaCl, 2.7 mM KCl) three times. Paraformaldehyde (4% in water, 500 μ l) was added and incubated for 20 min at room temperature to fix cells, followed by washing three times with PBS (500 μ l). Cells were incubated with Triton X-100 (0.2% in PBS, 500 μ l) for 5 min at room temperature, followed by washing with PBS (500 μ l) three times each for 5 min. Cells were incubated with blocking buffer (2% BSA, 2% normal goat serum, and 0.2% gelatin in PBS) for 1 h at room temperature, followed by incubation of cells with streptavidin-Cy5 (10 μ g/ml) in blocking buffer (50 μ l) for 30 min. Cells were washed with BSA (0.1% in PBS, 500 μ l) three times, each for 5 min. DAPI (100 μ g/ml in blocking buffer) was added to cells for 5 min, followed by washing with PBS (500 μ l) three times each for 5 min. Cover slips were immersed in water then fixed on a glass slide containing a solution of Mowiol® 4–88 saturated with DABCO (6 μ l), as described by the manufacturer (Sigma-Aldrich, MO, USA). The slide was incubated at 31°C for 30 min, then kept at 4°C until microscopy. An Olympus microscope (Tokyo, Japan) was used to monitor fluorescence signal using laser wavelengths

Figure 2. Cell permeabilization of ATP-biotin by deacetylated chitosan. (A) Fluorescence microscopy with DC. HeLa cells were incubated with ATP, ATP-biotin, DC or the ATP-biotin/DC complex, followed by washing, fixing and visualizing with SA-Cy5 to observe biotin (top) or DAPI to stain nuclear DNA (bottom). Repetitive trials are shown in Figure S1. (B) Fluorescence microscopy with Dig. HeLa cells were incubated with digitonin or the ATP-biotin/digitonin complex, followed by washing, fixing and visualizing with SA-Cy5 (top) and DAPI (bottom). Repetitive trials are shown in Figure S2. (C) MALDI monitoring of the ATP-biotin/DC complex. HeLa cells were incubated with ATP-biotin, DC or the ATP-biotin/DC complex, followed by analysis by MALDI-TOF MS. The ATP-biotin peak is at 934 *m/z*, whereas the 943 *m/z* peak is a matrix contaminant. Repetitive trials are shown in Figure S3. DC: Deacetylated chitosan; Dig: Digitonin; SA-Cy5: Streptavidin-Cy5.

corresponding to DAPI (excitation/emission maximum 358/461 nm) and Cy5 (excitation/emission maximum 678/694 nm). DAPI images were generated at 10 ms, whereas Cy5 images were generated at 500 ms. Three independent trials are shown in Figures 2A, 2B, S1 and S2.

MALDI-TOF *in cellulo* imaging of ATP-biotin

HeLa cells (20,000) were suspended in F-12 media containing 11% FBS, penicillin (9 units) and streptomycin (9 units; 50 μ l), and then added to a well of a 96-well plate. An equal volume (50 μ l) of final concentrations of ATP-biotin (4 mM), DC (3.75 mg/ml), or ATP-biotin/DC complex (4 mM/3.75 mg/ml) was added to separate wells and cells were incubated for 2 h at 37°C in a 5% CO₂ environment. As a control, one well without ATP analog was used as a reference. All reaction volumes were 100 μ l. Cells were collected by centrifugation at 1000 rpm for 5 min at 4°C, then washed twice with DPBS (100 μ l). Cell pellets were resuspended in water (20 μ l). The cell suspension (1 μ l) was mixed with a saturated solution of α -picolinic acid in 50% acetonitrile (1 μ l), and then applied to a MALDI plate (Bruker, MA, USA) for MS analysis. The MS spectra were analyzed in negative ion mode for the presence of ATP-biotin at *m/z* 934. Three

independent trials are shown in Figures 2C and S3.

Cell-viability assessment of DC

HeLa cells (100,000) were grown in a 12-well plate for 48 h in F-12 growth media (F-12 media containing 10% FBS, penicillin [9 units], and streptomycin [9 units]) at 37°C in a 5% CO₂ environment. Various concentrations of the ATP-biotin/DC complex (1 mM/0.9 mg/ml, 2 mM/1.88 mg/ml, 4 mM/3.75 mg/ml, 8 mM/7.5 mg/ml, or 10 mM/9.4 mg/ml) were prepared in F-12 growth media as described above. Each ATP-biotin/DC mixture was added separately to cells. Cells were incubated for 2 h at 37°C in a 5% CO₂ environment and then the complex was removed. Cells were washed twice with DPBS (400 μ l), followed by gentle scraping. Cells were collected by centrifugation at 4°C and 1000 rpm for 5 minutes, followed by resuspension in DPBS (100 μ l). Equal volumes of cell suspension and trypan blue (0.4% solution, Invitrogen, CA, USA) were mixed and cells were counted using a hemocytometer. As a control, untreated cells grown in the absence of ATP-biotin/DC were counted, as described. Percentage cell viability was calculated by dividing the number of ATP-biotin/DC-treated live cells by untreated ones, and then multiplying by

100. Kaleidagraph (Synergy Software) was used to calculate the EC₅₀ of ATP-biotin/DC using a sigmoidal binding curve $y = 100 / (1 + (x/EC_{50})^n)$. The results shown are from three independent trials (Figure S4A & B).

Cell viability assessment of digitonin

Cells were prepared and treated as described for DC treatment, except for the following. Cells were treated with ATP-biotin (4 mM) and digitonin (20 μ g/ml) in P buffer (400 μ l; 25 mM Tris-HCl, pH 7.5, 7.2 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4, 124 mM NaCl, 2.3 mM KCl, 2 mM dithiothreitol [DTT], and 10 mM MgCl₂) containing Xpert protease inhibitor cocktail (1X, GenDEPOT) for 1 h at 37°C in a 5% CO₂ environment. As a control, cells were also treated with ATP-biotin (4 mM) and digitonin (20 μ g/ml) in F-12 growth media (F-12 media, 10% FBS, penicillin [9 units], and streptomycin [9 units]) for 1 h at 37°C in a 5% CO₂ environment. Digitonin treatment was restricted to 1 h due to high toxicity at longer times. The results shown are from three independent trials (Figure S4C).

DC-assisted *in cellulo* kinase-catalyzed biotinylation of HeLa cells

HeLa cells (200,000) were prepared and treated in a 12-well plate as described above. The media was removed and then cells were incubated with ATP-biotin alone or the ATP-biotin/DC complex in F-12 growth media under the same cell growth conditions. To optimize the ATP-biotin/DC ratio, cells were incubated with DC alone (3.75 mg/ml) or different ratios of ATP-biotin/DC complex; 1:1 (4 mM/3.75 mg/ml), 2:1 (4 mM/1.88 mg/ml) and 1:2 (4 mM/ 7.50 mg/ml) in F-12 growth media for 2 h (Figure S6). To optimize the incubation time, the 1:1 ATP-biotin/DC complex (4 mM/3.75 mg/ml) was incubated for 30 min, 1 h and 2 h (Figure S7). The optimized conditions for all subsequent studies used ATP-biotin alone (4 mM) or the 1:1 ATP-biotin/DC complex (4 mM/3.75 mg/ml) in F-12 growth media with a 2-h incubation period. As a control, cells were preincubated with kinase inhibitor staurosporine (1 μ M) in F-12 growth media at 37°C in a 5% CO₂ environment for 1 h before incubating with the ATP-biotin/DC mixture. After the media was removed, cells were washed with DPBS (400 μ l) two times, harvested by scraping, and collected by centrifugation at 1000 rpm for 5 min at 0°C. Cells were lysed by resus-

Figure 3. Kinase-catalyzed biotinylation in live cells. (A) DC-assisted *in cellulo* kinase-catalyzed biotinylation of HeLa cells with ATP–biotin or ATP–biotin/DC complex under normal growth conditions. As a control, HeLa cells were preincubated with kinase inhibitor STSP (lane 4). Repetitive trials are shown in Figure S5A. (B) *In vitro* kinase-catalyzed biotinylation of HeLa lysates with ATP–biotin. As a negative control, ATP–biotin was incubated with heat-denatured HeLa cell lysates. Repetitive trials are shown in Figure S5B. (C) Digitonin-assisted *in cellulo* kinase-catalyzed biotinylation of HeLa cells with ATP–biotin under normal growth conditions in either buffer or media. Repetitive trials are shown in Figure S8. For all parts, SDS–PAGE gel analysis was used to separate reaction mixtures and gels were visualized with SA–Cy5 (top) or SYPRO® Ruby total protein stain (bottom). SA–Cy5: Streptavidin–Cy5; STSP: Staurosporine.

subsequent addition of ATP–Biotin (4 mM) and digitonin (20 μ g/ml) with staurosporine (1 μ M) in P buffer (400 μ l). As a comparative control, cells were treated with ATP–biotin (4 mM) and digitonin (20 μ g/ml) in F-12 growth media (400 μ l) for 2 h at 37°C. After incubation, the buffer or media was removed, cells were washed with DPBS (400 μ l) three times, harvested by scraping, and collected by centrifugation at 1000 rpm for 5 min at 0°C. Cell lysates were created and proteins were analyzed by SDS–PAGE as described above. Repetitive trials are shown in Figures 3C and S8.

Digitonin-assisted biotinylation of harvested cells in buffer

HeLa cells (2×10^6 cells) were harvested, washed with DPBS, and permeabilized for 5 min on ice with digitonin (50 μ g/ml) in P buffer (400 μ l). After permeabilization, the P buffer containing digitonin was removed and cells were washed once with cold DPBS (400 μ l). Cells were then treated with ATP–biotin (4 mM) in P buffer (400 μ l) for 2 h at 31°C with mild shaking at 100 rpm. As a control, cell pellets were incubated with P buffer (400 μ l) in the absence of digitonin before the ATP–biotin was added. After incubation, cells were washed with DPBS (400 μ l) three times, and cell lysates were created as described above. Proteins were visualized using SDS–PAGE analysis as described above. Repetitive trials are shown in Figure S9.

Kinase-catalyzed biotinylation of lysates

HeLa cells (200,000) were prepared and treated in a 12–well plate as described above [34]. Cells were collected and lysates were prepared as described above. Lysates were incubated with ATP–biotin (4 mM) for 2 h at 37°C in a total reaction volume of 20 μ l. As a control, ATP–biotin (4 mM) was incubated with heat denatured cell lysates, which were generated by heating the lysates to 95°C for 1 min. Proteins were visualized using SDS–PAGE analysis as described previously. Repetitive trials are shown in Figure 3B and S5B.

Results & discussion

To test if DC can promote the cell permeabilization of ATP–biotin, fluorescence microscopy studies were performed. ATP–biotin and DC were first preincubated to form an ATP–biotin/DC complex. The ATP–biotin/DC complex was then incubated with

pending in lysis buffer (21 μ l, 50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 10% glycerol, and 1X protease inhibitor cocktail [GenDepot]) and incubating on ice for 30 min with rocking. Lysates were collected by removing cell debris through centrifugation at 13,200 rpm for 10 min at 4°C. SDS–PAGE gel analysis (10%) was used to separate the proteins in the cell lysates and proteins were visualized by SYPRO® Ruby stain (ThermoFisher, MA, USA). Biotinylation was detected with a streptavidin–Cy5 conjugate (Life Technologies, CA, USA) after transferring onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, MA, USA). The gels were imaged using a Typhoon imager (GE

Healthcare Life Sciences, IL, USA) using excitation and emission wavelengths of 650 nm and 670 nm. Three independent trials are shown in Figure 3A and S5A.

Digitonin-assisted *in cellulo* biotinylation in buffer & media

HeLa cells (200,000) were prepared and treated in a 12–well plate as described above. Cells were then treated with ATP–biotin (4 mM) in the absence or presence of digitonin (20 μ g/ml) in P buffer (400 μ l) for 1 h at 37°C in a 5% CO₂ environment. To confirm labeling was kinase dependent, cells were preincubated with staurosporine (1 μ M) in P buffer (400 μ l) for 30 min at 37°C in a 5% CO₂ environment before removal and

HeLa cells under normal growth conditions, followed by washing, fixing and staining with SA-Cy5 to detect biotin. As a control, cells were treated with ATP-biotin alone. Using a 1:1 ratio of ATP-biotin:DC and 2 h incubation, only cells treated with the ATP-biotin/DC complex showed significant fluorescence signal (Figure 2A and S1). ATP-biotin alone in media showed only background fluorescence. As a comparative control, cells were also incubated with an ATP-biotin/digitonin complex. With the same concentration of ATP-biotin (2 mM) used in the DC-assisted permeabilization studies, ATP-biotin/digitonin showed only background fluorescence (Figure 2B and S2). These experiments indicated that DC promotes the cell permeability of ATP-biotin under normal cell growth conditions with high efficiency.

As a secondary method to confirm ATP-biotin cell delivery by DC, mass spectrometry (MS) analysis was used. HeLa cells were incubated with ATP-biotin or the ATP-biotin/DC complex, washed, and analyzed by mass assisted laser desorption ionization-time of flight (MALDI-TOF) MS. Only cells treated with the ATP-biotin/DC complex showed a peak at 934 *m/z* corresponding to ATP-biotin (Figure 2C and S3). Cells treated with ATP-biotin alone did not show the presence of the 934 *m/z* peak. These results confirm that DC promotes the cell permeability of ATP-biotin.

In preparation for cell labeling, we assessed the dose-dependent cytotoxicity of ATP-biotin/DC in HeLa cells. The ATP-biotin/DC complex showed an EC_{50} of 8.0 ± 0.6 mM/ 7.5 ± 0.6 mg/ml, respectively (Figure S4A). HeLa cells showed viability of $79 \pm 5\%$ for ATP-biotin/DC concentrations used in later kinase-catalyzed biotinylation reactions (4 mM/3.75 mg/ml, Figure S4B). For comparison, we also tested the cytotoxicity of ATP-biotin/digitonin and observed only $55 \pm 3\%$ cell viability at the concentrations used in later biotinylation reactions (4 mM/20 μ g/ml, Figure S4C). Taken together, these studies indicated that DC is less cytotoxic than digitonin at concentrations needed for cell permeabilization.

Having confirmed the cell permeability and low toxicity of DC, kinase-catalyzed biotinylation in live cells was tested. HeLa cells growing in culture were incubated with the ATP-biotin/DC complex, followed by washing, harvesting, lysis and separation of proteins by SDS-PAGE. Biotinylation was

observed in cells treated with the ATP-biotin/DC complex (Figure 3A and S5A, lane 5), which is consistent with cell permeability. As a control, HeLa cells pretreated with the kinase inhibitor, staurosporine, showed reduced biotinylation (Figure 3A and S5A, lane 4), which confirmed that biotinylation is kinase dependent. ATP-biotin alone did not show labeling (Figure 3A and S5A, lane 2), which indicated that DC is required for cell permeability. Although several ATP-biotin:DC ratios were tested (Figure S6), maximal labeling and minimal precipitation were obtained with a 1:1 ratio of ATP-biotin:DC, which is consistent with prior reports studying DC-assisted cell permeability [33]. Biotinylation was observed after 30 min, 1 h and 2 h incubation periods (Figure S7), although a 2 h incubation time was selected to be consistent with earlier work in lysates. These experiments establish that DC-permeabilization is compatible with live-cell labeling.

As a comparative control, we tested the ability of digitonin to promote live-cell kinase-catalyzed labeling. Using similar conditions to the DC permeabilization studies, cells were incubated with an ATP-biotin/digitonin mixture under normal growth conditions and then analyzed by gel methods. No biotinylation was observed (Figure 3C, lane 13 and Figure S8, lane 5), indicating low efficiency of permeabilization. Similar results were also observed when cells were incubated with an ATP-biotin/digitonin mixture in buffer (Figure 3C, lane 14 and Figure S8, lane 6) [35]. As a further comparison, harvested cells were digitonin-permeabilized in buffer using published conditions [20]. In this case, cells were harvested, washed permeabilized with digitonin in buffer, and then incubated with ATP-biotin in buffer. With harvested cells, biotinylation of cellular proteins was observed (Figure S9, lanes 4 and 8), although at lower levels compared with DC-assisted biotinylation (Figure 3A and S5A, lane 5). These results document that DC permeabilization offered greater labeling efficiency under normal cell growth conditions compared with digitonin permeabilization.

Most experiments with ATP analogs have been performed in lysates, not live cells, due to the lack of permeability. With a method for ATP permeabilization, we wondered if studies with ATP analogs in live cells would show significant differences compared with lysate-based experiments. To compare in cell with *in vitro*

kinase-catalyzed biotinylation, ATP-biotin was incubated with HeLa cell lysates and biotinylated proteins were analyzed by gel methods (Figure 3B and S5B). The extent of in-cell DC-assisted biotinylation (Figure 3A and S5A, lane 5) compared with *in vitro* labeling (Figure 3B and S5B, lane 8) was different. In particular, lysate biotinylation produced different labeled bands compared with *in cellulo* labeling. The differences observed with DC-assisted in-cell biotinylation compared with lysate labeling are consistent with earlier studies using an intrinsically cell-permeable ATP-biotin analog [27]. The difference in biotinylation in live cells versus lysates might be due to cellular compartmentalization, which suggests that ATP-biotin labeling in live cells will better reflect the cellular phosphoproteome than lysate-based labeling.

In conclusion, this study documents use of DC to permeabilize ATP analogs for cell-based studies. Compared with other methods, DC-assisted permeabilization offers several advantages. DC can be generally applied to permeabilize any ATP analog, which avoids synthesis of intrinsically cell-permeable ATP derivatives. Importantly, DC permeabilization is compatible with normal cell growth conditions for studies in a physiologically relevant environment. In fact, a comparison of live-cell versus lysate labeling documented differences in the quality of biotinylation, suggesting that in-cell studies will be valuable. DC permeabilization represents an exciting and enabling advance to study cell signaling using ATP analogs under physiologically relevant conditions.

Author contributions

AEF performed all experiments with DC, except Figures S6 and S7, which were performed by VR. DME performed all experiments with digitonin. MKHP conceived the project and assisted in the experimental design and interpretation. All authors contributed to writing the manuscript.

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Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2018-0076

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