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Protein Delivery: If your GFP (or other small protein) is in the cytosol it will also be in the nucleus

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Abstract

Intracellular protein delivery is a transformative tool for biologics research and medicine. Delivery into the cytosol allows proteins to diffuse throughout the cell and access subcellular organelles. Inefficient delivery caused by endosomal entrapment is often misidentified as cytosolic delivery. This inaccuracy muddles what should be a key checkpoint in assessing delivery efficiency. Green fluorescent protein (GFP) is a robust cargo small enough to passively diffuse from the cytosol into the nucleus. Fluorescence of GFP in the nucleus is a direct readout for cytosolic access and effective delivery. Here we highlight recent examples from literature for the accurate assessment of cytosolic protein delivery using GFP fluorescence in the cytosol and nucleus.

Introduction

Protein therapeutics are powerful tools for targeted manipulation of cellular processes.[1] Proteins are responsible for modulating cellular activities like autophagy and homeostasis, and protein dysfunction can cause disease on a cellular or organismal level.[2-4] Controlled introduction of functional proteins into the cell is a promising strategy to alleviate disease symptoms.[5,6] Further, cytosolic delivery of exogenous proteins provides a key tool for the fundamental study of protein function in cells.[7,8]

Entry into the cytosol is a critical checkpoint for the assessment of most protein therapeutics.[9,10] From the cytosol, proteins can access subcellular organelles, apoptotic machinery, and the nuclear membrane.[11,12] Direct entry into the cytosol is generally barred to proteins by the selectively permeable cell membrane.[13]

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A number of nanocarriers[14,15] and cell-penetrating peptides[16,17] have been engineered for delivery of proteins into the cell. However access to the cytosol is often limited in efficiency because these systems are generally uptaken by endocytosis[18] through a range of mechanisms.[19] Endocytic uptake entraps particles in vesicles (endosomes) that sequester them from the cytosol, ultimately leading to either endo/lysosomal degradation or exocytosis.[20,21] Choice of carrier material, formulation, and treatment conditions are critical considerations for effective delivery, and all contribute to both cellular uptake and intracellular distribution.[22-24] Engineered delivery vehicles can promote endosomal 'escape' through various methods of endosomal rupture, [25-28] but recent reports estimate that even utilizing these methods <10% of endosomally delivered cargo can be expected to enter the cytosol.[29,30] Recently, vehicles capable of non-endosomal uptake through direct cytosolic entry have been reported with highly efficient protein delivery, providing a potential alternative pathway[31,32] Distinguishing cytosolic access from endosomal entrapment however, is a critically important yet often overlooked checkpoint in the development of effective intracellular delivery systems.[23,33]

Fluorescent proteins provide a straightforward and effective readout to evaluating cytosolic access. The integral fluorescence of these proteins eliminates artifacts arising from intracellular separation of the fluorophore from the protein cargo.[34,35] Green fluorescent protein (GFP) is a robust and versatile model cargo that can passively diffuse from the cytosol into the nucleus through pores in the nuclear membrane.[36] Nuclear fluorescence of GFP thus provides definitive evidence of cytosolic access, making it a versatile tool for evaluating intracellular protein delivery (Figure 1). In this topical review we will discuss the use of both GFP and fluorophore-tagged small proteins as tools for validating cytosolic protein delivery, highlighting fundamental concepts and recent applications of this strategy.

Diffuse Fluorescence Indicates Cytosolic Access

There are two main indicators of cytosolic access of small fluorescent (or fluorophore-tagged) proteins that are easily evaluated by fluorescence microscopy: punctate *versus* diffuse fluorescence signal and fluorescence signal in the nucleus. Diffuse fluorescence qualitatively defines endosomal entrapment from cytosolic access. Fluorescence in the nucleus provides a direct readout, as small proteins like GFP will diffuse from the cytosol through nuclear membrane pores and illuminate the nucleus.

Fluorescent proteins trapped in endosomes are visible as highly localized, punctate fluorescence signals. In contrast fluorescent proteins free to move about the cytosol display diffuse, evenly spread fluorescence, as seen in cells that constitutively express fluorescent protein. [37] Recently, Gao reported a method allowing small proteins to avoid endocytosis and pass into the cytosol through noncovalent tagging with Coomassie blue (CB)-cholesterol conjugates.[38] Cytosolic access of four dye-labeled proteins was assessed by diffuse *versus* punctate fluorescence signal (Figure 2) as well as co-localization with LysoTracker. The two smaller proteins were delivered to the cytosol, as evidenced by diffuse fluorescence throughout the cytosol. Lysozyme displayed a mixture of diffuse and punctate fluorescence. Notably, the small size of these proteins also allowed them to diffuse across the nuclear

pore and into the nuclei. The larger proteins showed only localized punctate fluorescence, indicating vesicular entrapment.

Co-localization of protein fluorescence with an endosomal marker is a common method to validate endosomal entrapment.[39] A common misconception is that protein cargo not co-localized with endosomal marker has avoided the endosome and entered the cytosol, even if fluorescence signal remains punctate. Endosomal markers like those of the LysoTracker series[40] work through pH-responsiveness, making them specific for early (pH ~6.3) or late stage (pH 4.5 - 5) endo/lysosomes.[33] When LysoTracker dyes aggregate, intracellular pH also rises considerably, potentially quenching the fluorophore of the delivery cargo.[41,42] Lysotracker co-localization should therefore always be qualified by fluorescence signal to accurately assess endosomal escape. Flavell recently reported a method to quantify endosomal escape of fluorescently labelled saporin toxin in Daudi cells using flow cytometry. [43] Endosomal escape was induced by treatment with chloroquine, an inhibitor of endolysosomal acidification. Pulse width analysis was used to distinguish punctate signal from diffuse cytosolic fluorescence. A fluorescence shift from punctate to diffuse corresponded with protein escape from the endosome, providing a metric of cytosolic access.

GFP in the Cytosol will Enter the Nucleus

GFP is an ideal model cargo to visualize protein trafficking into and throughout the cell. The fluorophore of GFP is composed of amino acid residues within the polypeptide chain, providing robust fluorescence (quantum yield up to 80 percent)[44] while avoiding the issues of photobleaching or enzymatic cleavage associated with fluorescent dyes. [45-47] Fluorescence is only lost upon denaturation, which can be used as a readout for retention of protein structure through the delivery process.

In addition to the diffuse fluorescence discussed above, GFP also provides a reliable readout of cytosolic access through nuclear fluorescence. [48] The nuclear membrane pores limit entry by passive diffusion to approximately 60kDa.[49,50] Small proteins like GFP (238 residues, 27 kDa) will passively translocate from the cytosol into the nucleus through these size-restrictive pores. [51] For proteins larger than the nuclear pore, passive nuclear diffusion is dramatically decreased and generally requires the aid of active nuclear targeting elements.[52, 53] Nuclear fluorescence therefore provides a direct readout of cytosolic access, as endosomally entrapped GFP can access neither the cytosol nor the nucleus. In one example of this effect, Raines modified GFP allowing it to pass through the cell membrane and into the cytosol by cloaking its carboxyl groups with a hydrophobic moiety.[54] Modified GFP showed diffuse fluorescence throughout the cytosol and nucleus. Delivery was compared with a super-charged GFP variant, which displayed dark nuclei and punctate signal indicative of endocytosis (Figure 3). In this study deliveries were performed through incubation for 30, 120, or 240 min at 37 °C in serum-free culture media.

We recently reported an imaging flow cytometric method that utilized the passive translocation of GFP into the nucleus as a quantitative indicator of cytosolic delivery. Cytosolic delivery efficiency was quantified through co-localization of GFP signal with

nuclear stain. Supramolecular nanocomposite vehicles were generated between engineered oligo-glutamate, or E-tagged GFP and guanidinium-functionalized poly(oxanorbornene) imide (PONI) polymers. The charge ratio between guanidinium (polymer) and E-tag (protein) during formulation was found to be critical to nanocomposite generation. These vehicles efficiently delivered GFP directly to the cytosol, with diffuse cytosolic and nuclear fluorescence (Figure 4).[55] Notably these vehicles demonstrated cytosolic delivery in the presence of serum (media supplemented with 10% fetal bovine serum). Under the most efficient delivery conditions, GFP was delivered to the cytosol in >90% of the cell population as indicated through GFP fluorescence in the nucleus. This simple and straightforward approach to quantifying cytosolic delivery is widely applicable to small fluorescent or dye-labeled protein delivery, regardless of carrier vehicle.[56-59]

Conclusions

In sum, GFP and fluorophore-tagged proteins provide two key readouts for cytosolic access. Diffuse fluorescence provides a simple readout but is challenging to quantify in a cell population. Diffuse nuclear fluorescence definitively demonstrates cytosolic access of delivered GFP and can be quantified in cell populations using microscopy and imaging flow cytometry. These robust, versatile methods are suitable for fluorophore-tagged small proteins, and using fluorescent proteins like GFP further eliminates artifacts arising from cargo degradation and fluorophore release. Widespread utilization of these tactics provides rigorous assessment of cytosolic access that will promote the development of more efficient platforms for protein delivery.

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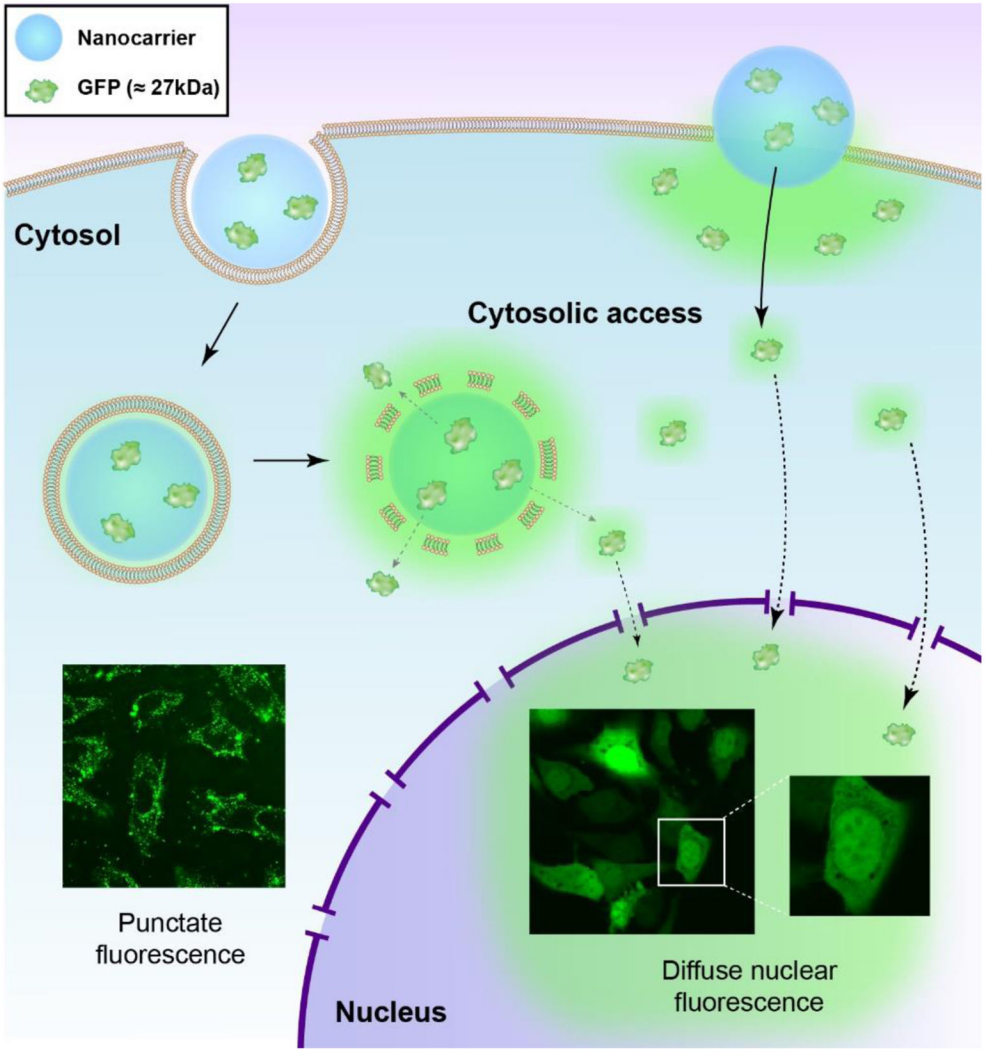

Figure 1.

Protein nanocarriers are uptaken by the cell through endocytosis *(left)* or direct cytosolic entry *(right)*. Endocytosis presents as punctate fluorescence in the cytosol. Diffuse fluorescence suggests cytosolic access. Small proteins (< 60kDa) like GFP can passively diffuse from the cytosol into the nucleus, definitively establishing cytosolic access.

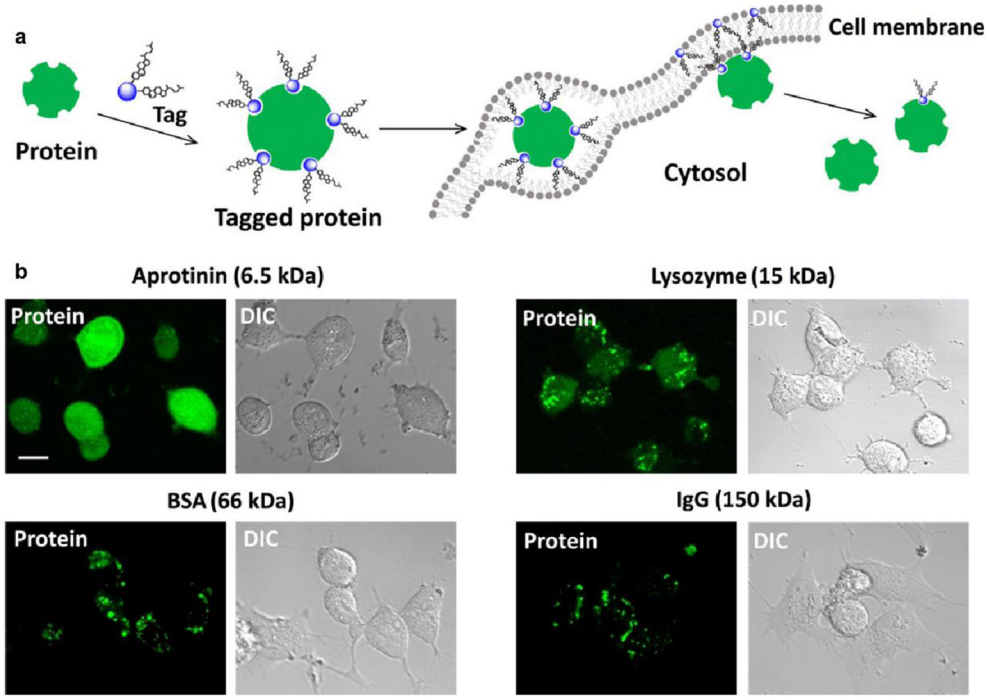

Figure 2.

Cytosolic delivery by protein tagging. (a) Proposed delivery mechanism. Proteins tagged with CB-cholesterol conjugates embedded within the lipid bilayer. The noncovalent nature of the tagging causes dissociation, after which untagged hydrophilic protein can ‘slip’ into the cytosol. Deliveries were performed in serum-free RPMI 1640 media for 3 hours. (b) Confocal fluorescence and bright-field micrographs of live HeLa cells treated with tagged proteins of different molecular weights. All proteins were labeled with a green fluorescent dye (AF488). Adapted with permission from [35].

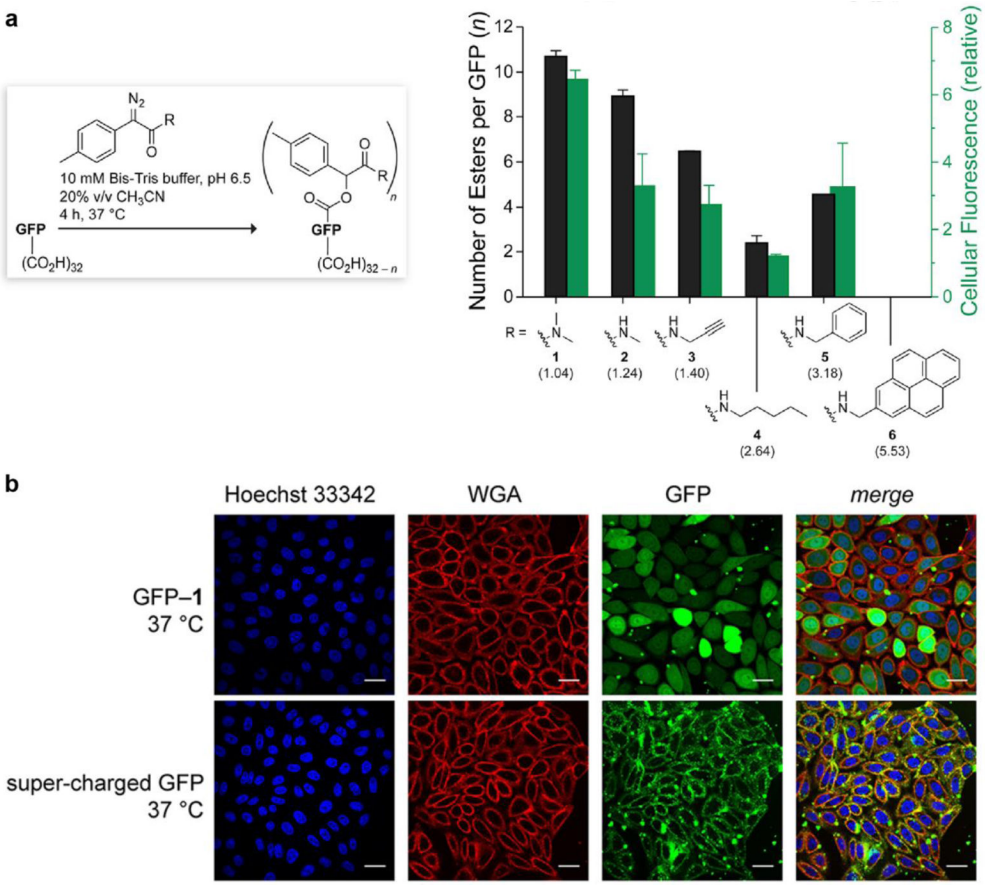

Figure 3.

(a) Bar graph showing the extent of esterification of the superfolder variant of GFP with diazo compounds 1–6 (black) with parenthetical log *P* values, and the internalization of the ensuing esterified GFPs into CHO-K1 cells (green). Values (±SD) were determined by mass spectrometry and flow cytometry, respectively. (b) Images of the cellular internalization in CHO-K1 cells of esterified GFP (*top*) and its supercharged variant *(bottom*). Cells were stained with Hoechst 33342 and wheat germ agglutinin (WGA)–AlexaFluor 647. Scale bars: 25 μm. Adapted with permission from [51].

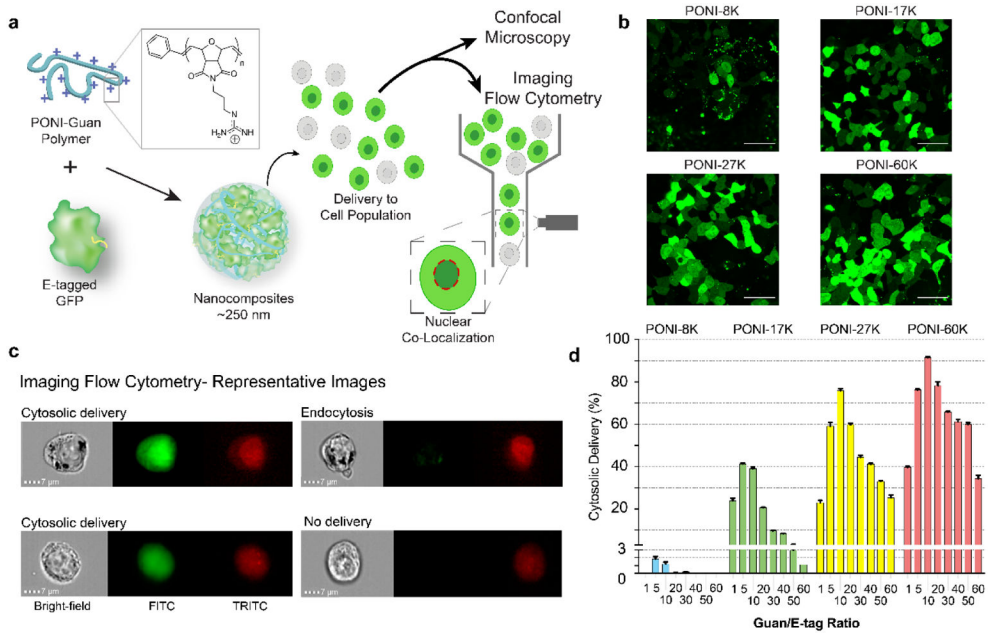

Figure 4.

Polymer mediated intracellular delivery of GFP, quantified by imaging flow cytometry. (a) PONI polymer electrostatically complexed with ‘E-tagged’ GFP to form supramolecular polymer-protein nanocomposites. Delivered cell population was analyzed by confocal microscopy or imaging flow cytometry, through co-localization with a nuclear stain. (b) Confocal images of GFPE20 showing cytosolic delivery and diffuse nuclear fluorescence in HEK-293T cells. Scale bars = 50 μm. (c) Representative imaging flow cytometry micrographs showing cytosolic delivery and nuclear localization of GFPE20 as compared to endosomally entrapped GFP and undelivered cells. Channels displayed are bright-field, FITC for GFP, and TRITC for nuclear stain (DRAQ5). (d) Percentage of cell population with cytosolic delivery of GFP using a family of different molecular weight PONI polymers. Adapted with permission from [52].