Multiplexed Quantitative Screening of the Cellular Uptake of Proteins Delivered by Polymeric Nanocarriers

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Abstract

Proteins are important therapeutic agents, yet better methods are needed to deliver them inside cells. Polymeric nanocarriers (PNCs) are versatile materials for this purpose, and to enhance their development, it is necessary to quantify protein delivery efficiency into cells by numerous PNC designs at the same time. Current strategies for screening PNC systems are qualitative and mostly serial. Here, we describe a multiplexed approach that uses metal-coded mass tags (MMTs) to quantify protein delivery into cells by several different PNC designs simultaneously. Our approach will facilitate the development of more potent delivery systems by improving precision and reducing costs, effort, and time.

Therapeutic proteins constitute a market approaching ~$400 billion, with hundreds of candidates approved and in clinical trials.[1] Unlike small molecule drugs, proteins can perform a broad range of biological functions with high specificity and potency.[1] Despite their enormous potential, they have some limitations, including poor membrane permeability,[2,3] immunogenicity,[4] and susceptibility to degradation,[5] which pose significant challenges to their clinical development.

Nanocarriers (NCs) have proven to mitigate protein immunogenicity, augment their stability, and enhance their cellular uptake.[6-9] Various nanocarriers, including liposomes,[10] virus-like particles,[11] polymers,[12] and metal-based materials[13] have been developed. Polymeric NCs (PNCs) stand out as promising candidates for protein delivery due to their facile synthesis, tunable structures,[14,15] extended circulation times,[16] and potential for stimuli-responsive drug release.[17] However, the large design space of PNCs necessitates parametric optimization to enhance their protein delivery capabilities.[18] During parametric optimization, it would be valuable to quantitatively assess protein uptake into cells by many PNC designs at the same time to identify the most effective ones. Such a multiplexed quantitative method currently does not exist for PNCs, yet creating such a method would facilitate development of better delivery systems by reducing time and cost, while improving measurement precision.

Fluorescence-based methods, such as confocal microscopy and flow cytometry, are commonly used assays to monitor the intracellular delivery of proteins that are inherently fluorescent or dye-tagged.[19,20] These approaches are broadly applicable, yet simultaneously measuring multiple proteins is challenging due to interferences from different emission channels.[21] When it comes to quantitation, fluorescence methods face inherent challenges, including background interferences, quenching, and environment-dependent fluorescence emission.[22,23]

Liquid chromatography coupled with tandem mass spectrometry (MS) can be used to

quantify proteins in complex biological samples,[24,25] with the most reliable quantification involving internal standards, but the need for such standards can limit the number of proteins studied at the same time.[26] Mass cytometry has also emerged as a valuable MS-based strategy, using lanthanide (Ln)-labeled antibodies to simultaneously measure more than 40 proteins;[27-29] however, antibodies are required for each detected protein, making the measurement indirect and more costly.

In this study, we describe metal-coded mass tags (MMTs)[30] that quantify the cellular delivery of proteins by several distinct polymeric nanomaterials at the same time, achieving better measurement precision and throughput because of the inherent advantages of multiplexing. PNCs with different characteristics are each loaded with an MMT-conjugated protein, enabling, to our knowledge, the first-ever multiplexed quantitation of protein uptake by different PNCs. Our approach promises to yield insight into the relationship between PNCs physiochemical properties and their delivery efficiencies (Figure 1). As a model delivery system, we use previously reported polymeric-protein nanocomposites (PPNCs) that are formulated by mixing guanidinium-functionalized poly(oxanorborneneimide) (PONI) polymers as the PNC and polyglutamate-tagged enhanced green fluorescent protein (EGFP) as the model cargo.[20] We demonstrate that protein delivery by up to 9 different PPNCs can be quantified at the same time. However, the methods described here could be expanded to even higher levels of multiplexing (~40) using available lanthanides and their isotopes.

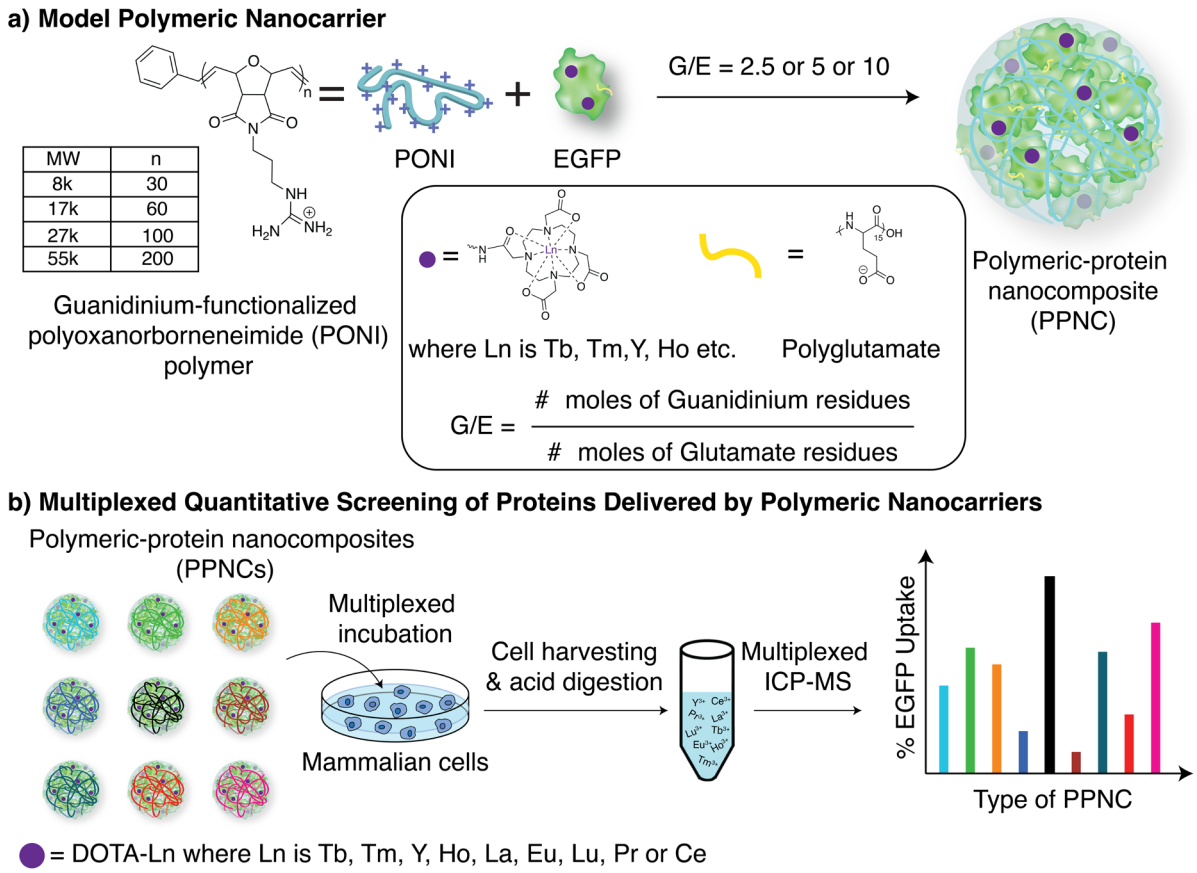

Figure 1. Metal-coded mass-tagged (MMT) polymeric-protein nanocomposites (PPNCs) for quantitative measurements of the cellular uptake of the protein EGFP as a model system. (a) Formulation strategy used in this work. (b) Workflow for analyzing the cellular uptake of proteins delivered by PPNCs in a single experiment using ICP-MS.

To quantify protein delivery by multiple PPNCs, we synthesized and used polymers with different molecular weights.[20] EGFP with a 15-glutamate (E-15) tag at the C-terminus was recombinantly expressed, as described previously,[20] and conjugated with different MMTs (Figure 2) to surface-exposed lysine residues via reactions with N-hydroxy succinimide-derivatized dodecane tetraacetic acid (DOTA-NHS). After conjugation with the DOTA group, metal complexation was accomplished with the appropriate $LnCl_3$ salt of interest (see Supplemental Experiment Section in the SI for details), and free metal was removed via three washes and centrifugal filtration with a 10 kDa molecular weight cutoff (MWCO) filter (see Figure S1a). The number of Ln metal ions per protein was determined both by electrospray ionization mass

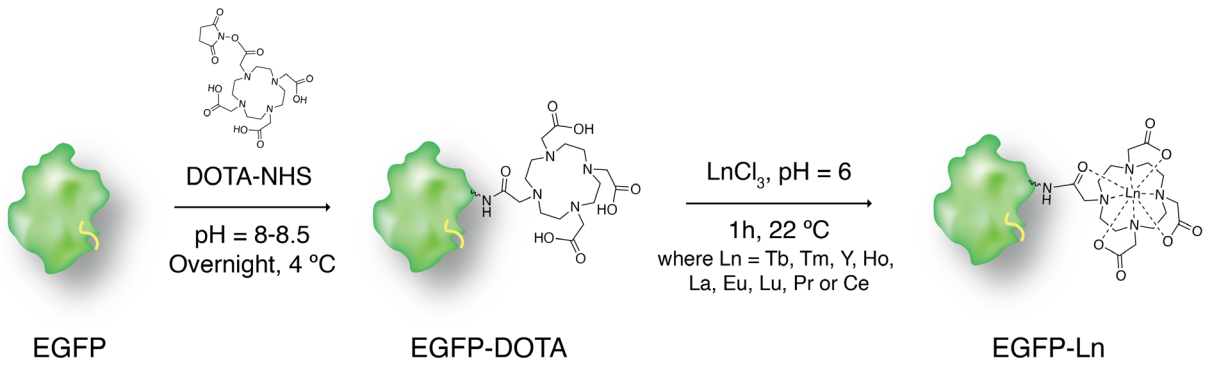

Figure 2. Schematic illustrating conjugation of MMTs to E-tagged EGFP. The conjugation was achieved through DOTA-NHS conjugation and then Ln^{3+} metal ion complexation.

spectrometry (ESI-MS) measurements (Figure S1b, c) and by ICP-MS (Table S1). The PPNCs were formulated[20] by mixing the metal-tagged EGFP with the polymer of interest for 10 min before dilution with either cell culture media or phosphate-buffered saline (PBS). The ratio of guanidinium (G) moieties on the polymer to the glutamate (E) residues (i.e. G/E ratio) on the EGFP protein was set at either 2.5, 5, or 10 (Figure 1a). PPNC size was determined by dynamic light scattering, and charge was confirmed by zeta potential measurements (Figure S2). Results indicate that the PPNC are 100s of nm in size, and the assemblies with G/E ratios of 2.5 and 5 are anionic, whereas the assemblies with a G/E ratio of 10 are neutral.

As an initial evaluation of our strategy for quantifying protein uptake, two separate PPNC formulations having equal amounts of a 17k or a 55k polymer were assembled with EGFP-Eu and EGFP-Tb, respectively, at a G/E ratio of 10. These assemblies were separately incubated with HEK293T cells (see Supplemental Experiment Section in the SI for details). After 24 h of incubation, the cells were pelleted, washed three times with cold PBS to remove non-specifically bound proteins on the outside of the cells (Figure S3), acid-digested [*Note: - the acid mixture used is highly corrosive and must be used with caution*], and analyzed by ICP-MS on a PerkinElmer NexIon 300X (see Supplemental Experiment Section in the SI). Response factors for Eu and Tb (Figure S4a) were generated and used to convert the corresponding metal ion signals into moles

of protein (Figure S4b). The amount of EGFP taken up during triplicate incubations of the 17k polymer was 0.59 ± 0.03 pmol for ~500,000 cells, corresponding to 3.3 ± 0.2 % of the protein added to the cell culture. For the 55k polymer, 1.4 ± 0.3 pmol was taken up by the cells, which corresponds to 1.8 ± 0.4 % of the protein added to the cell culture.

To assess the accuracy of our ICP-MS results, we compared them to previous work in which the same PPNC systems were studied by flow cytometry. To do so, we prepared four separate PPNC formulations (Table S2), each of which incorporated equal amounts of 8k, 27k, or 55k polymers and an MMT-conjugated protein at a G/E ratio of 5 or 10. The protein delivery efficiencies of these formulations into HEK293T cells were then measured using ICP-MS. The results revealed that PPNCs with 55k polymers having a G/E ratio of 5 or 10 (i.e. 55k-5 and 55k-10) delivered the highest amount of EGFP protein, whereas the PPNCs with an 8k polymer at a G/E ratio of 5 (i.e. 8k-5) delivered the lowest amount of EGFP protein (Figure S5a). These results are consistent with prior findings measured by flow cytometry (Figure S5b),[20] indicating that the MMT approach provides reliable information about protein uptake into cells.

Next, to demonstrate the multiplexing capability of the MMT approach, the cellular uptake of four different PPNC formulations was concurrently studied. First, to ensure that polymers and proteins from one PPNC formulation do not undergo crosstalk with other PPNC assemblies, a dialysis-based assay was used (see Supplemental Experiment Section in the SI for details). For this assay, a PPNC with Tm-tagged EGFP was assembled with a Tb-tagged 27k polymer, so that both protein and polymer could be quantified by ICP-MS. The polymer was tagged by creating a diblock copolymer with guanidinium and amine blocks at a 9:1 ratio.[31] The amine block of the polymer was conjugated with a Tb-containing MMT as described previously (see Supplemental Experiment Section in SI and Figure S6).[30] The assembled PPNC, having a G/E ratio of 10, was loaded into a

dialysis tube with a MWCO of 100 kDa (Figure S7). After 24 h of incubation in PBS, the amounts of polymer and EGFP that crossed the dialysis tube membrane were quantified by using ICP-MS. Approximately 1% of the PONI polymer crossed the dialysis membrane, while no detectable EGFP crossed the dialysis membrane (Table S3). Interestingly, polymers without any loaded protein also have about ~1% of the polymer cross the dialysis membrane. These results suggest that multiplexing measurements are feasible as the extent of crosstalk between assemblies is minimal (~ 1%) after 24 h.

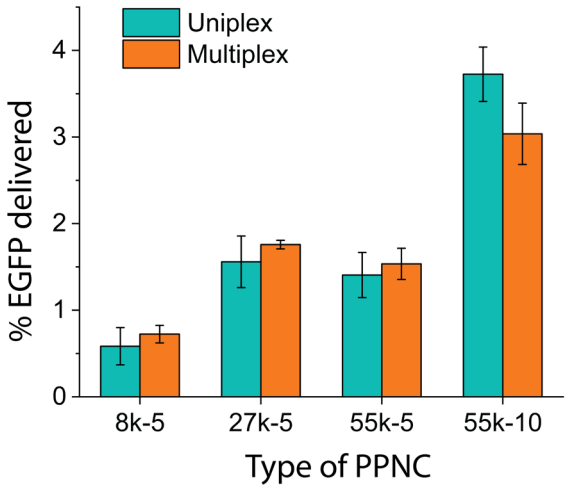

Figure 3. Validation of MMT-assisted multiplexed cellular uptake quantification of PPNC-delivered protein. Four PPNCs (see Table S2) were incubated with HEK293T cells. After 24 h, EGFP delivered by each of the four PPNCs was quantified in a single experiment and compared with the uniplexed analyses where each PPNC was individually spiked into HEK293T culture. Error bars represent the standard deviations of three replicate experiments (n=3).

After ensuring minimal crosstalk between assemblies, four different PPNC formulations (i.e. 8k-5, 27k-5, 55k-5, and 55k-10) were mixed in cell culture media for multiplexing. The multiplexed PPNCs were incubated with HEK293T cells for 24 h, and the percent delivery of each MMT-conjugated EGFP was measured by ICP-MS. These multiplexed results were then compared with uniplexed PPNC cell uptake results (Figure 3). The similarity in EGFP delivery between the uniplexed and multiplexed measurements demonstrates that MMTs can be effectively utilized to screen multiple PONI-based PPNC formulations simultaneously.

Next, nine distinct PPNC formulations, each containing equal amounts of EGFP but varying amounts of PONI polymers at three different G/E ratios (i.e. G/E = 2.5 or 5 or 10) (Table

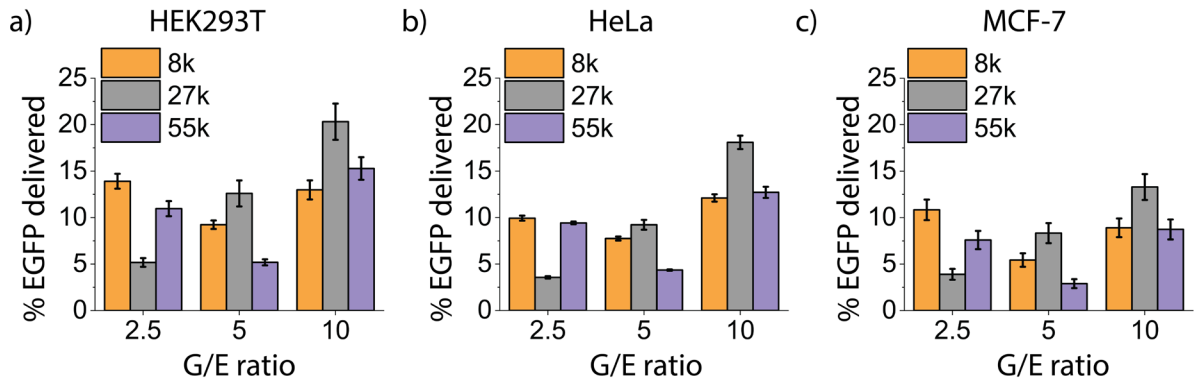

Figure 4. Multiplexed screening of PPNCs for their EGFP delivery efficiency. A mixture of nine different PPNCs (each with equal amounts of EGFP) was spiked into (a) HEK293T (b) HeLa, and (c) MCF-7 cells. After 24 h of incubation, the quantities of EGFP delivered by each of the nine PPNCs were quantified in a single experiment. The error bars represent the standard deviations of three replicate experiments.

S4) were co-incubated for 24 h with three cell lines, HEK293T, HeLa, and MCF-7 cells, to test the versatility of the method. After quantifying protein uptake (Figure 4a-c), some interesting observations are apparent. First, the amount of protein delivered is not substantially influenced by polymer molecular weight, as there is no clear trend in uptake for the 8k, 27k, or 55k polymers. Instead, the G/E ratio appears to more significantly influence protein uptake, with a ratio of 10 generally providing the most efficient protein delivery, particularly for the 27k and 55k polymers (see Table S5 for statistical comparisons). Assemblies with G/E ratios of 2.5 and 5.0 have a net negative charge, whereas assemblies with a G/E of 10 are neutral, as exemplified for the 55k polymers. Generally, anionic nanomaterials penetrate the cell membrane of nonphagocytic cells, like the ones studied here, less efficiently than neutral or cationic nanomaterials,[32] perhaps explaining the higher uptake of assemblies with a G/E ratio of 10. The trends in protein delivery are similar for the different cell types, although the extent of uptake is lowest for the MCF-7 cells.

Overall, our results here demonstrate that MMTs, together with ICP-MS analysis, can be used to quantify the cellular uptake of proteins as delivered by PNCs. Importantly, protein delivery can be quantified for multiple PNCs at the same time by taking advantage of the ability of ICP-

MS to measure different lanthanide metals simultaneously. Our preliminary multiplexed screening indicates that protein delivery is mostly independent of PONI polymer molecular weight, but it is somewhat dependent on the G/E ratio for high molecular weight PONI polymers, which likely reflects the net charge characteristics of the assemblies. Looking ahead, using this approach to investigate protein delivery by PNCs *in vivo* would reduce the number of animals needed, decrease biological variability, and enable a better understanding of how nanocarrier structure affects their performance.

Author Contributions

D.K.A. and R.W.V conceived the idea, designed the experiments, and wrote the manuscript; D.K.A. performed the MMTs conjugations, *in vitro* experiments, ICP-MS characterizations, and dialysis experiments. R.G. provided the polymers and Y.A.C. provided the EGFP protein. R.G. and Y.A.C. assisted with cell culture experiments. N.T. characterized the PPNCs.

Supporting Information

Materials used in the experiments; the synthetic protocol for DOTA and metal conjugation; dialysis experiments for assessing polymer/protein crosstalk; cell culture experimental details; calculation of the moles of protein in the cells; figures showing the characterization of the protein conjugation; size and zeta potential measurements results; optimization of washing steps to remove free metal; calibration curves for quantification; comparison of ICP-MS and fluorescence results; synthetic scheme for polymer conjugation with DOTA and metal; tables indicating characterization of different metal-protein and PPNC formulations; and statistical analyses of cellular uptake.

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