Endoprotease-Mediated Intracellular Protein Delivery Using Nanocapsules

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Statement of Purpose: Intracellular delivery of active proteins is an essential therapeutic goal in various medical applications, including cancer therapy, imaging, vaccination, and treating loss of functioning genes in many diseases.[1] However, most native proteins are unable to penetrate the cell membrane and often suffer from loss of function due to proteolysis or aggregation in serum. Using an enzymatic-based method for intracellular delivery would achieve a level of specificity and control which is challenging with current methods and we have recently reported a specialized method using polymeric nanocapsules (NCs).[2] In order to deliver functional proteins that can interact with different cellular targets, a general mechanism for degradation of the polymer capsule and release of the protein cargo is needed. Thus we sought to design the NC that can disintegrate and release proteins in response to the actions of the essential endoprotease furin, which is a ubiquitous proprotein convertase.[3] Furin is localized in various intracellular locations and has a preferred substrate in the form RX(K/R)R.[4] Furin processes a diverse group of endogenous proproteins and foreign substrates. Notably, furin-mediated cleavage of the papillomavirus minor capsid protein L2 is necessary for dissociation of the capsid, release of viral DNA and subsequent infection.[5] Inspired by these natural roles of furin in facilitating cargo release, we sought to design a furin-degradable biomimetic protein delivery vehicle.

Methods: We synthesized a furin-degradable bisacrylated crosslinker peptide (Ahx-RVRRSK) by solid phase peptide synthesis on rink amide resin using differential acid labile protection groups. To prepare NCs, co-monomers acrylamide and *N*-(3-aminopropyl) methacrylamide and crosslinker were adsorbed onto the surface of the target protein. This was followed by *in situ* free radical interfacial polymerization to form the polymeric shell and assemble NCs. We performed protein release studies both in cell-free systems and *in vitro* which were visualized with confocal and transmission electron microscopy and quantified with ELISA.

Results: We noncovalently encapsulated protein cargo in a thin, positively charged polymer layer, which is crosslinked by furin-cleavable peptides and observed compact spherical 10-15 nm NCs with TEM which completely dissociated after 10 h incubation with furin (Fig1AB). To further confirm the furin-degradable nature of NCs, we quantified the release of eGFP from NCs using ELISA (Fig1C). Significant eGFP release was observed upon incubation of furin-degradable NCs with furin which was dramatically attenuated upon addition furin inhibitor, dec-RVKR-cmk to levels of furin-degradable NCs without furin and non-degradable NCs crosslinked with *N,N'*-methylene bisacrylamide. We then sought to deliver proteins to the nuclei of mammalian cells and first chose eGFP fused to the nuclear localization signal PKKKRKV (NLS-eGFP) and delivered protein NCs to Chinese hamster ovary (CHO) cell lines with varied intracellular furin concentrations. We observed significant nuclear localization of eGFP in the wild-type CHO-K1 and furin-overexpressing FD11+furin cell lines while eGFP was confined to the cytosol in furin deficient FD11 cells (Fig2A). This indicates that upon entry into cells where furin activities are abundant, the crosslinkers are proteolyzed and the polymeric matrix degrades, leading to the release of native protein. Confocal microscopy observations were confirmed with ELISA on nuclear fractions of treated cells (Fig2B) which collectively indicates that the presence of active intracellular furin and a furin-degradable NC are both required for successful delivery. We next explored internalization of NCs in human cell lines. First, we established the ability of NCs to escape from the early endosomes to the cytosol in HeLa cell lines. We also demonstrated delivery of the anticancer caspase-3 (CP3) to HeLa cells using furin-degradable NCs and confirmed the bioactivity of CP3 remains intact by the TUNEL assay. We have also been able to deliver NLS-eGFP to the nuclei of human amniotic-fluid derived cells which can differentiate into all three germ layers and have significant therapeutic potential. We have also delivered the transcription factor Klf4 to the nuclei of mouse embryonic fibroblast cells and observed more efficient nuclear uptake by NCs compared to 11R-tagged Klf4 using immunocytochemistry.

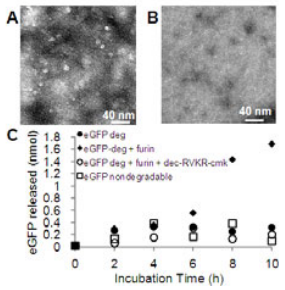
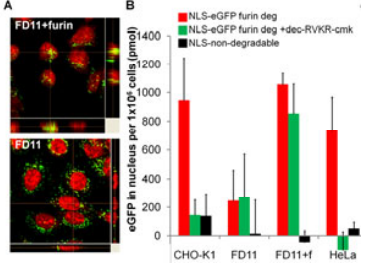


Fig 1: **NC Characterization**

Fig 2: Intracellular Delivery to CHO cells



Conclusions: We successfully demonstrated both cytosolic and nuclear delivery of proteins using our engineered NC carrier which degrades in response to the ubiquitous endoprotease furin. Different cell lines were demonstrated to be amenable hosts for delivery including the immortalized HeLa, the highly regenerative hAFDC, and the essential structural MEF. We also showed that protein cargos of different sizes and tertiary structures can be encapsulated and released reversibly without loss of bioactivity, including 27 kDa beta barrel eGFP; 51 kDa Klf4 with three zinc finger regions; and 64 kDa heterotetramer CP3 . In summary, through extensive imaging and quantitative analysis *in vitro,* we have shown the successful delivery of both cytoplasmic and nuclear proteins based on specific furin-mediated degradation and cargo release. This approach may also be applicable to the other therapeutics.

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