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Self-assembled protein nanocarrier for intracellular delivery of antibody

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ABSTRACT

Despite the great potential of antibodies as intracellular therapeutics, there is a significant, unmet challenge in delivering sufficient amounts of folded antibodies inside cells. We describe an all-protein self-assembled nanocarrier capable of delivering functional antibodies to the cytosol. By combining an α-helical peptide that self-assembles into a hexameric coiled-coil bundle and an Fc-binding Protein A fragment, we generated the Hex nanocarrier that is efficiently internalized by cells without cytotoxicity. Localization of multiple Fc-binding domains on the hexameric core allowed the Hex nanocarrier to tightly bind antibody with sub-nanomolar affinity regardless of pH and the antibody's originating species. The size of the Hex nanocarrier ranges from 25 to 35 nm depending on the antibody loading ratio. We demonstrated the capacity of the Hex nanocarrier to deliver functional antibodies to the cytosol by employing anti-β-tubulin or anti-nuclear pore complex antibody as cargo. The design of the Hex nanocarrier is modular, which enables functionalization beyond Fc-binding. We exploited this feature to improve the cytosolic delivery efficiency of the Hex nanocarrier by addition of an endosomolytic motif to the core. The modified Hex nanocarrier exhibited similar antibody-binding behavior, but delivered more antibodies to their cytosolic targets at a faster rate. This work demonstrates an efficient intracellular antibody delivery platform with significant advantages over existing approaches as it does not require modification of the antibody, is biodegradable, and has an antibody to carrier mass ratio of 13, which is greater than other reported antibody carriers.

1. Introduction

Protein engineering and recombinant technologies have created new classes of protein therapeutics with bioactivities not possible from small molecules [1]. A particular success is antibodies, which have been engineered to bind a number of extracellular targets and are a significant and growing fraction of the protein therapeutics portfolio [2]. These have shown incredible promise in the clinic for a variety of applications including inflammation, regenerative medicine, cancer, and autoimmune disease [3–5]. Despite the successes of antibodies, the most notable exception from the current list is those with intracellular targets or function. The reason is not a lack of medical need. There is a significant list of "undruggable" targets, disease related protein-protein interactions that small molecules cannot block [6]. The issue is not that antibodies cannot be found or engineered to bind these targets, as antibody engineering is a powerful tool and already many antibodies exist for intracellular targets but they are only used on fixed cells in laboratory immunostaining assays. The intrabody, an intracellularly expressed antibody, has been developed to target cellular components in live cells, but requires *in vivo* genetic manipulation and delivery for therapeutic applications [7]. Arguably, the primary reason there are no intracellular antibody therapeutics is the significant, unmet challenge in delivering sufficient amounts of functional protein inside cells. Successful design and implementation of a functional intracellular antibody delivery system that is generally applicable to all antibodies would have significant impact on human health as it would open the door for a host of new antibody drug therapies to be realized for a wide variety of diseases.

Approaches in the limited intracellular antibody delivery literature mirror those in the more expansive intracellular protein delivery literature and include individual antibody modification, application of traditional small molecule delivery carriers, and novel types of antibody nanocarriers. Immunoglobulin G (IgG) has been covalently modified with polyarginine and demonstrated intracellular delivery and function *in vitro* [8], but use of non-covalent cationic HIV-TAT peptide or cationic lipids have shown only *in vitro* delivery with no assessment of function [9,10]. Small antibody mimics, such as monobodies, have been modified with anthrax toxin components which enable intracellular delivery *in vitro* [11]. Individual modifications like these can increase cellular uptake of functional antibodies or mimics, but it is not clear if single antibody delivery will be sufficient *in vivo* and some cationic modifications have toxicity concerns. Traditional small molecule delivery nanoparticles suffer from extremely low antibody loading.

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Polymersomes deliver functional IgG intracellularly *in vitro*, and model (non-functional) IgG *in vivo* but the max theoretical loading of 0.015 IgG molecules per polymersome is not practical [12,13]. Polylactic-*co*-glycolic acid nanoparticles also have 1% or less IgG loading and intracellular delivery and function has not been shown [14]. Novel antibody nanocarriers have promise, but protein loading per mass of carrier is still low and there can be issues with the material safety, degradability, or clearance profiles. Gold nanoparticles and quantum dots have been covalently associated or affinity-coated with up to 10 IgG molecules and demonstrated intracellular delivery *in vitro*. However, they are meant for imaging experiments [15,16]. IgG has also been adsorbed to silica nanoparticles, which induces intracellular uptake and function *in vitro*, though the loading is only 1.26 μg IgG/mg silica [17]. Virus-like particles (VLPs) have been employed to encapsulate a higher load of antibodies for intracellular delivery, but immunogenic concerns should be addressed for clinical applications [18,19]. While there are disadvantages to the current approaches, they importantly demonstrate that the reducing intracellular environment does not prevent cytosolic antibody function.

In this work we create an antibody carrier that combines specific protein-protein interactions and nanoparticle benefits. The nanocarrier is made entirely from protein, a self-assembled hexameric barrel that displays antibody affinity binding sites. The theoretical loading is 6 antibodies per hexamer, resulting in more antibody mass than carrier mass. Also, the antibody-bound carrier is large enough to take advantage of active nanoparticle uptake mechanisms to achieve increased internalization over soluble antibody. The design is general, capable of carrying any antibody or antibody fragment with an Fc domain. This approach will create the therapeutic connection between the significant number of undruggable intracellular targets and antibodies that can be engineered for those targets. Though we test proof of concept in HeLa cells with antibody delivery to cytosolic antigens including beta tubulin and nuclear pore complex, a wide range of human diseases could be treated with intracellular antibodies given an effective delivery carrier.

2. Materials and methods

2.1. Materials

The following primary antibodies were purchased from Abcam (Cambridge, MA): mouse anti-beta tubulin antibody, ab135209; mouse anti-nuclear pore complex proteins antibody, ab50008. The fluorescently labeled secondary antibody, goat anti-mouse IgG Alexa Fluor 647 conjugate (A-21235), was purchased from Thermo Scientific (Waltham, MA). Purified IgG from mouse serum and rabbit serum were obtained from Equitech-Bio (Kerrville, TX) and Sigma-Aldrich (St. Louis, MO), respectively. Biotin-PEG_4-NHS ester and TAMRA-NHS ester were obtained from Bioconjugate Technology Company (Scottdale, AZ). Glass bottom dishes were purchased from Cellvis (Mountain View, CA). MTT cell viability assay kit was purchased from Biotium (Hayward, CA). PD-10 desalting columns were obtained from GE Healthcare (Piscataway, NJ). Vivaspin centrifugal concentrators with MWCO of 10 kDa were obtained from Sartorius Corporation (Bohemia, NY). Mammalian cell culture media, antibiotics, fetal bovine serum (FBS), phosphate buffered saline (PBS) were purchased from Thermo Scientific. The bacterial expression vector (pQE-80), Ni-NTA agarose beads and DNA miniprep kits were obtained from Qiagen (Valencia, CA). The streptavidin (SA)-coated biosensors were obtained from Pall ForteBio LLC (Menlo Park, CA). Other chemicals were purchased from Sigma-Aldrich.

2.2. Cloning and bacterial expression of fusion peptides

The gene construct encoding H6-SPAB-Hex or Hex-SPAB-H6 was synthesized by GenScript (Piscataway, NJ) and subcloned into an IPTG-inducible bacterial expression vector pQE80, yielding pQE80-SPAB-Hex and pQE80-Hex-SPAB, respectively. Additional sequence information is described in the Supplementary Table S1. The bacterial expression host, *Escherichia coli* TOP10, was transformed with the pQE80-SPAB-Hex or pQE80-Hex-SPAB, and was grown in 2 × YT medium at 37 °C. Protein expression was induced by 1 mM IPTG when the OD reached 0.6, and continued for 5 h. After collecting cells by centrifugation, proteins were purified by Ni-NTA affinity chromatography under native conditions according to the manufacturer's instruction. For expression of the SPAB domain (H6-SPAB) alone, the Hex domain including the flexible linker sequence between SPAB and Hex was deleted by the restriction-free cloning method [20] using pQE80-SPAB-Hex as a template, resulting in pQE80-SPAB. For expression of the Au-Hex nanocarrier, the restriction-free cloning method was used to insert the Aurein 1.2 domain N-terminally and C-terminally to pQE-Hex-SPAB and pQE-SPAB-Hex, respectively. A vector encoding H6-SPAB-Hex-TAT-HA2 was similarly constructed. Transformation, expression and protein purification were performed in the same way as described above.

2.3. Generation and characterization of the Hex nanocarrier

Sodium dodecyl sulfate (SDS) was added to a final concentration of 0.2% (w/v) to a 1:1 M ratio mixture of purified H6-SPAB-Hex (1.5 mg/mL) and Hex-SPAB-H6 (1.5 mg/mL) in PBS. After 10 min, the mixture was buffer-exchanged to PBS using a PD-10 desalting column. The resulting Hex nanocarrier was concentrated using Vivaspin centrifugal filters and analyzed by SDS-PAGE using the Bio-Rad protein electrophoresis system. The particle size and zeta potential of the Hex nanocarrier in the absence or presence of antibody were measured by Malvern Zetasizer Nano ZS equipped with a 4 mW He-Ne laser light source (633 nm) at a protein concentration of 0.2 mg/mL. In order to evaluate the binding kinetics of the Hex nanocarrier towards antibodies, mouse or rabbit IgG dissolved in PBS at a concentration of 7 mg/mL (47 μM) was mixed with a 10-fold molar excess of Biotin-PEG_4-NHS at RT for 0.5 h, and then desalted using a PD-10 column to remove residual reagents. The biotinylated IgG was then immobilized onto the SA-coated biosensors using the Octet Red96 system according to the manufacturer's protocol (Pall ForteBio LLC), and then subjected to the binding kinetics assays with various concentrations of the Hex nanocarrier or the SPAB domain at pH 7.4 (PBS) or 5.0 (0.1 M sodium phosphate). By using the Octet Data Analysis software, binding kinetics sensorgrams were processed, and fitted to a simple 1:1 binding model to determine various kinetic parameters.

2.4. MTT assay

HeLa cells were seeded onto a 96-well plate at a density of 5000 cells per well in 100 μL DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 μg/mL penicillin and 50 μg/mL streptomycin), and incubated overnight for attachment at 37 °C under humidified air containing 5% (v/v) CO_2. The medium was replaced with 100 μL fresh medium containing a protein(s) of interest at appropriate concentrations and/or molar ratios. Cells were incubated at 37 °C for 24 or 48 h. After replacing the protein-containing medium with 100 μL MTT solution (10 μL MTT stock plus 90 μL DMEM/FBS), cells were incubated at 37 °C for 4 h, and then mixed with 200 μL DMSO containing 0.01 N HCl to dissolve the water-insoluble metabolic product, formazan. After 30 min incubation at 37 °C, the absorbance was read at 570 nm by a microplate reader.

2.5. Cellular uptake of fluorescently labeled antibodies by the Hex nanocarrier

Mouse or rabbit IgG dissolved in PBS (pH 8.5) at the concentration of 7 mg/mL (47 μM) was mixed with a 10-fold molar excess of TAMRA-NHS at RT for 1 h, and then desalted using a PD-10 column to remove