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Design and cellular internalization of genetically engineered polypeptide nanoparticles displaying adenovirus knob domain

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Abstract

Hepatocytes and acinar cells exhibit high-efficiency, fiber-dependent internalization of adenovirus; however, viral capsids have unpredictable immunological effects and are challenging to develop into targeted drug carriers. To exploit this internalization pathway and minimize the use of viral proteins, we developed a simple gene product that self assembles nanoparticles decorated with the knob domain of adenovirus serotype 5 fiber protein. The most significant advantages of this platform include: (i) compatibility with genetic engineering; (ii) no bioconjugate chemistry is required to link fusion proteins to the nanoparticle surface; and (iii) it can direct the reversible assembly of large nanoparticles, which are monodisperse, multivalent, and biodegradable. These particles are predominantly composed from diblock copolymers of elastin-like polypeptide (ELP). ELPs have unique phase transition behavior, whereby they self-assemble above a transition temperature that is simple to control. The diblock ELP described contains two motifs with distinct transition temperatures, which assemble nanoparticles at physiological temperatures. Analysis by non-denaturing-PAGE demonstrated that the purified knob-ELP formed trimers or dimers, which is a property of the native knob/fiber protein. Dynamic light scattering indicated that the diblock copolymer, with or without knob, is able to self assemble into nanoparticles ~40 nm in diameter. To examine the functionality of knob-ELP, their uptake was assessed in a hepatocyte cell-line that expresses the receptor for adenovirus serotype 5 fiber and knob, the coxsackievirus and adenovirus receptor (CAR). Both plain ELP and knob-ELP were bound to the outside of hepatocytes; however, the knob-ELP fusion protein exhibits more internalization and localization to lysosomes of hepatocytes. These findings suggest that functional fusion proteins may only minimally influence the assembly temperature and diameter of ELP nanoparticles. These results are a proof-of-principal that large fusion proteins (>10 kDa) can be assembled by diblock ELPs without the need for bioconjugate chemistry, which greatly simplifies the design and evaluation of targeted drug carriers.

Keywords

Elastin like polypeptides; nanoparticles; knob; cellular uptake; hepatocyte

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Introduction

Since discovered a half century ago, human adenovirus has attracted attention because different types cause significant levels of respiratory, ocular, and gastrointestinal disease [1]. Because of its pathological effects, a significant amount of information is therefore available on its mode of interaction with cells. Adenovirus serotype 2 and serotype 5 within subgroup C, the best understood types of this virus, attach to cells through the initial binding of the fiber protein to the cell-surface coxsackievirus and adenovirus receptor (CAR) [2, 3]. CAR is a 46-kDa high affinity receptor that is present in many human tissues, including liver, heart, lacrimal gland, lung, and brain [4, 5] and which is thought to function as a cell adhesion protein [6]. Upon surface binding, adenovirus entry in most cells is facilitated through interaction of an additional adenoviral capsid protein, the penton base, with integrin receptors on the plasma membrane, a process facilitating efficient entry via endocytosis [7]. Intriguingly, a penton-independent mechanism for internalization of Ad5 fiber and fiber-derived peptides including the knob domain has been described in acinar cells [5] and also seems to exist in hepatocytes [8]. This mechanism, which occurs in cells expressing the most abundant CAR protein in the body, has been reported to deliver a region of the fiber capsid protein, the knob, to a subcellular degradative compartment [5, 8]. After entry, through either fiber/knob- or penton-dependent interactions, mechanisms have been described for the subsequent interactions between the virus and other intracellular transport machinery, which facilitate efficient trafficking of the viral DNA to the nucleus. For these and other reasons, most notably the ability of adenovirus to transduce non-dividing cells, adenovirus serotype 5 and other serotypes have been explored as vectors for gene therapy [9]. However, despite their relatively efficient cellular endocytosis and gene transfer, viral vectors in general have intrinsic drawbacks, such as limited opportunities for repeat administrations due to acute inflammatory responses and delayed humoral or cellular immune responses [10]. In addition, some viral vectors integrate DNA into the genome, resulting in insertional mutagenesis [11].

In the past few decades, numerous research groups have focused on drug carriers, such as liposomes, micelles, dendrimers, and polymersomes [12]. Relatively few drug carriers have been approved for use in humans, which suggests that better strategies and materials may be required to generate successful nanomedicines. One emerging strategy is to design genetically engineered protein polymers that self-assemble directly into nanoparticles [13]. For example, the elastin-like-polypeptides (ELP) are a genetically engineered polypeptide with unique phase behavior [14], which promotes recombinant expression, protein purification and self-assembly of nanostructures [15]. Genetically engineered ELPs are biodegradable and biocompatible [16]. ELPs are composed of the repeated amino acid sequence (Val-Pro-Gly-Xaa-Gly)_n, where the hydrophobicity of Xaa determines the polypeptide phase behavior. In this manuscript, ELPs are identified by the single letter amino acid code of the guest residue followed by the number of repeat units, *n*. For example, S48I48 represents a diblock copolymer ELP with 48 serine (S) pentamers at the amino terminus and 48 isoleucine (I) pentamers at the carboxy terminus.

Our group was the first to report the unique fiber-dependent internalization mechanism for adenovirus type 5 in lacrimal acinar epithelial cells, and to report that this internalization mechanism can be recapitulated by the knob domain of the fiber protein [5]. This mechanism seems to operate in hepatocytes as well to enable internalization of free knob protein to intracellular degradative compartments [8]. This is in contrast to other demonstrations that fiber is responsible only for the initial binding of adenovirus to the cell via CAR, and not for internalization, which is driven by the penton base capsid protein [7] and integrin receptors on the plasma membrane, a process facilitating efficient entry [7, 17]. Some studies have recently shown that CAR, which is a cell adhesion protein and thought to

be largely surface associated, can in fact be endocytosed [18]. Altogether these studies suggest that in certain cells, such as acinar cells and hepatocytes where CAR is highly abundant, that a subpopulation may serve as an internalization receptor when bound to fiber or knob proteins. The physiological relevance of this endocytotic population of CAR in these cells is so far unknown. To exploit this apparent CAR internalization pathway and high affinity interaction with viral proteins for drug delivery while minimizing the use of the entire viral capsid, we here report the development of a simple gene product that assembles nanoparticles decorated with the knob domain of adenovirus fiber protein. The most significant advantages of this platform include: (i) compatibility with genetic engineering; (ii) no bioconjugate chemistry is required to link fusion proteins to the nanoparticle; (iii) and the resulting polypeptides assemble into nanoparticles that are monodisperse, multivalent, and biodegradable. These particles are predominantly composed of diblock copolymers of ELP. ELP block copolymers self-assemble multimeric nanoparticles above a transition temperature that can be controlled by adjusting their hydrophobicity and molecular weight (Fig. 1). Above the critical temperature for the ELP diblock copolymer, the knob-ELP fusion protein also assembles nanoparticles. Similar to adenovirus, our hypothesis is that nanocarriers displaying the knob domain may exhibit selective internalization into tissues expressing unique CAR-dependent endocytosis of fiber and knob. This manuscript describes the biophysical properties and cellular uptake of a knob-ELP, which self assembles nanoparticles that have potential applications for drug delivery and gene therapy.

Materials and Methods

Materials and Reagents

TB dry powder growth medium was purchased from MO BIO Laboratories, Inc. (Carlsbad, CA). NHS-Rhodamine was purchased from Thermo Fisher Scientific (Rockford, IL). Thrombin CleanCleave™ Kit, Polyethylenimine, Copper Chloride and insulin were obtained from Sigma-Aldrich (St. Louis, MO). The knob domain gene sequence was ordered from Integrated DNA Technologies (Coralville, IA). LysoTracker Green CN 26 was purchased from Invitrogen (Carlsbad, CA). Goat anti-mouse CAR antibody was obtained from R&D Systems (Minneapolis, MN). IRDye®800-conjugated donkey anti-goat second antibody was purchased from Rockland (Gilbertsville, PA). Blocking buffer was purchased from Li-COR Biosciences (Lincoln, NB). The transformed mouse hepatocyte cell line (PTEN loxp/loxp Alb/Cre -) was a kind gift from Dr. Bangyan L. Stiles, University of Southern California. The QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit were purchased from Qiagen (Valencia, CA).

Knob-ELP vector design

The plasmids encoding ELP were designed similarly to those reported previously [15, 19]. The knob domain gene sequence was designed with restriction enzyme NdeI and BamHI at 5' and 3' of the knob gene respectively. A thrombin amino acid recognition site (GLVPRGS) was incorporated between the knob sequence and the ELP sequence. A recognition site for BseRI was also incorporated to facilitate the insertion of ELP genes with complementary two base pair 5' overhang(s) (Supplementary Fig. 1). The plasmids containing genes that encode for ELP and knob were double digested by BseRI and BssHII, and the DNA pieces containing ELP and knob were purified using a gel extraction kit and then ligated together (Supplementary Fig. 1). Successful clones were confirmed by diagnostic DNA digestion, DNA sequencing, and mass spectrometry of the polypeptide gene products.

Purification of ELP fusion proteins

E. coli strain BLR (Novagen Inc., Milwaukee, WI) was transformed with the modified pET-25(+) expression vectors containing the ELP or knob-ELP genes. The bacteria were

grown overnight in 5 mL TB dry medium supplemented with 1 µg/mL ampicillin in an orbital shaker at 37 °C. Then bacteria were centrifuged down, and the pellet was resuspended in 2 liters TB dry medium and cultured for 24 hours in an orbital shaker at 37 °C. The bacteria were again harvested by centrifugation at 4 °C and resuspended in phosphate buffer saline (PBS). The bacteria were lysed by discontinuous pulsed ultrasonication in an ice-water bath. The insoluble debris was removed from the lysate by centrifugation and nucleic acids were precipitated by adding polyethylenimine (0.5% w/v final concentration) and removed by centrifugation at 4 °C. From the clarified bacterial lysates, the ELPs and knob-ELPs were purified by inverse transition cycling (ITC), which has been described previously [20–22]. Briefly, ELP solutions were warmed at room temperature and NaCl was added (1–3 M final concentration) to induce the ELP phase separation. The aggregated ELP fusion polypeptides were separated from the lysate by centrifugation at room temperature. The ELP pellet was resolubilized in PBS within an ice-water bath. The resolubilized ELP solution was centrifuged at 4 °C to remove remaining aggregated proteins. It was previously reported that purification cycles were repeated for two to six rounds as needed to purify various ELP fusion proteins [20, 23]. In this study, the purification cycle was repeated five times to remove nearly all of the contaminating *E. coli* proteins, which was essential because contaminants may aggregate during heating and bias the hydrodynamic radius. The purity of purified knob-ELP was measured using SDS-PAGE in a 10 % gel. After electrophoresis, the gel was stained with Coomassie brilliant blue.

Characterization of Knob-ELP

As described above, the knob-ELP was designed as a fusion protein consisting of a knob domain and an ELP. To study these multifunctional polypeptides, they were characterized by non-denaturing PAGE, turbidometric analysis of their temperature-dependent phase behavior, and dynamic light scattering. Native fiber/knob proteins are trimeric; therefore, the ability of knob-ELPs to self-associate was characterized using non-denaturing PAGE. Knob-S48I48 and recombinant knob was mixed with sample buffer without 2-mercaptoethanol or SDS, and then loaded onto a 10 % polyacrylamide gel without SDS at 4°C. At this temperature, the ELP nanoparticles remain dissociated, which enables the polypeptides to enter the gel. After three hours of electrophoresis, the gel was stained with Coomassie brilliant blue.

To explore the temperature-dependent phase behavior of the ELPs, optical density and hydrodynamic radius were observed over a range of temperatures. Knob-S96, S48I48 and knob-S48I48 were diluted to 25 µM in PBS on ice and the absorbance at 350 nm was monitored with a DU800 UV-Vis spectrophotometer (Beckman Coulter, Brea, CA) at a temperature gradient of 1 °C/minute. For dynamic light scattering studies, S48I48 and knob-S48I48 were diluted to 25 µM in PBS and passed through 20 nm membrane filters at 4 °C, and BSA, a protein with a similar molecular to knob-S48I48 was used as a control. Then 90 µL sample of was transferred into a 384 well microplate and covered with 20 µL mineral oil. The microplate and mineral oil were pre-chilled at 4 °C at least for 1 hour. The microplate was centrifuged at 4 °C to remove air bubbles from samples before and after addition of mineral oil. Then the sample was measured in a DynaPro plate reader (Wyatt Inc., Santa Barbara, CA) at temperature intervals of 1 °C. The resulting hydrodynamic radii were collected and analyzed by Dynamics (Wyatt Inc., Santa Barbara, CA). The measurements were repeated three times and particle radius for BSA, S48I48 and knob-S48I48 at 15 °C and 37 °C were analyzed by a one-way analysis of variance ($R^2=1.000$, $p=10^{-20}$, $n=18$).

Thrombin cleavage of knob-ELP

A thrombin recognition site was designed between the knob domain and ELP sequence (Table 1), which was cleaved by thrombin (Sigma-Aldrich, St. Louis, MO). Thrombin

immobilized on agarose beads was centrifuged to remove the storage buffer and washed with cleavage buffer (from the thrombin cleavage kit). The knob-S48I48 was then diluted with cleavage buffer to 1 mg/mL and suspended with thrombin agarose slurry for 24 hours at room temperature. After incubation, the thrombin agarose beads were removed by centrifugation. The cleaved knob (21.7 kD) was resolved by SDS-PAGE and gels were stained with Coomassie brilliant blue. The SDS-PAGE gel was scanned with a Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, CA) and analyzed with software Quantity one (Bio-Rad).

Conjugation with NHS-Rhodamine

To track cellular uptake, the ELPs S48I48 and knob-S48I48 were conjugated with NHS-Rhodamine (Thermo Fisher Scientific Inc, Rockford, IL) via covalent modification of primary amines at the amino end of the peptide. For S48I48, the only available amine is at the amino terminus; however, knob-S48I48 has an additional 11 lysine residues that may be sites of modification. The conjugation was performed in 100 mM borate buffer for 2 hours at 4 °C, and the conjugated ELP was separated by size exclusion chromatography on a PD10 desalting column (GE Healthcare, Piscataway, NJ).

Cellular uptake of knob-ELP/ELP

Hepatocytes were expected to be enriched in the CAR receptor, according to previous reports [5]. To confirm this, a western blot was performed on a murine hepatocyte cell-line. CHO cells were used as a negative control [2]. 2×10^4 cells were mixed with SDS-PAGE sample buffer and heated above 95 °C for 5 minutes. CAR was detected by western blotting using a goat anti-mouse CAR antibody as the primary antibody and IRDye®800-conjugated donkey anti-goat antibody as the secondary antibody. The result was scanned using an Odyssey® Imaging System (Li-COR, Lincoln, NE, USA) and quantified with the Odyssey® 1.1 software.

To observe uptake into mouse hepatocytes, cells were cultured on 35 mm glass coverslip-bottomed dishes with medium [(DMEM (4.5 g/L) containing 10% fetal bovine serum, 5 µg/ml insulin, and 0.02 µg/ml epidermal growth factor)]. Uptake studies were conducted when hepatocytes reached 70% confluence. After washing with warm fresh medium, hepatocytes were cultured in medium containing 10 µM of either S48I48 or knob-S48I48 conjugated with rhodamine, and 75 nM LysoTracker green. After 30 minutes incubation at 37 °C, the cells were rinsed with warm fresh medium to remove the free knob-ELP/ELP in the medium. Next cells were incubated with 75 nM LysoTracker green in a 37 °C incubation chamber. The chamber is mounted on a Zeiss LSM 510 Meta confocal microscope system, which is equipped with argon and red and green HeNe lasers and mounted on a vibration-free table.

To demonstrate the specificity of knob-ELP internalization for the CAR pathway, hepatocytes were pre-bound with goat anti-mouse CAR antibody. After confirming that the goat anti-mouse CAR antibody has a high affinity with the mouse hepatocytes (Supplementary Fig. 2), The anti-mouse CAR antibody (0.2 mg/mL) was diluted with warm medium 10-fold and incubated with the hepatocytes for 30 minutes at 37 °C. Knob-S48I48 conjugated with rhodamine was then added to the medium at a concentration of 10 µM. After 30 minutes incubation with knob-S48I48 and 75 nM LysoTracker green, the hepatocytes were rinsed, then incubated in fresh warm medium with 75 nM LysoTracker green and imaged as described above.

Results

Knob-ELP purification

A series of ELPs were genetically engineered, expressed in *E. coli*, and purified using the ELP temperature-dependent phase transition property (Table 1). The purified material was characterized for molecular weight and purity using SDS-PAGE and matrix assisted laser desorption ion mass spectrometry (Fig. 2, Table 1). Three fusion peptides with knob were prepared, knob-S96, knob-S48I48, and knob-I96. The ELPs S96 and I96 have a high and low transition temperature respectively; however, they do not form nanoparticles (data not shown). In contrast, the ELP S48I48 was shown to form nanoparticles at physiological temperatures (Table 1). Each of these fusion peptides appears as a major band around 60 kD (Fig. 2), which corresponds to the predicted and observed molecular weights as determined using mass spectrometry (Table 1). Some contaminating *E. coli* proteins appear to co-purify with both knob I-96 and knob-S96 but not knob-S48I48. Based on this data, it appears that the purity of knob-ELP fusion proteins, in particular knob-S48I48, is similar to that observed in previous reports [24, 25]. Although not essential for this study, the non-chromatographic purification of proteins fused to ELPs represents a powerful advantage of this approach.

Characterization of knob-ELP

To determine if the knob-ELP fusion peptides exist in a trimeric form, as they do for native adenovirus as required for appropriate CAR binding [26], non-denaturing gel electrophoresis was performed (Fig. 3). Knob-S48I48 surprisingly showed three strong bands around 60 kD, 120 kD and 180 kD, which indicated monomer, dimer and trimer forms of knob-ELP. For comparison, a recombinant knob purified using nickel affinity chromatography (without ELP) was also confirmed to form predominantly trimers [27, 28]. The recombinant knob lane indicates several minor bands, with molecular weights slightly lower than knob. These minor bands may come from partial proteolysis of knob. This data suggests that the ELP architecture may influence the native quaternary structure of fused proteins domains, whereby block copolymers that assemble nanoparticles (S48I48) also promote formation of native trimers. So the recombinant knob-ELP has properties similar to those of the native knob.

To characterize the ELP behavior of the knob fusion peptides, the transition temperatures were identified by optical density (Fig. 4) and the assembly of nanoparticles was confirmed using dynamic light scattering. Knob-S96, a monoblock ELP, only exhibits one increase in optical density over a temperature gradient at a temperature well above physiological conditions. Knob-I96 also shows a single increase in optical density; however, due to the hydrophobicity of the isoleucine Xaa residue, this fusion peptide phase-separates near room temperature. In contrast, the diblock ELP Knob-S48I48 displayed two phase transition temperatures, one around 19.5 °C and another around 60 °C. Qualitatively, this behavior is similar to S48I48, which has two transition temperatures at 26.5 and 75 °C. For knob-S48I48, at temperatures below 19.5 °C, the polypeptides are free in solution; however, between 19.5 and about 40 °C the peptides are presumed to form nanoparticles. Above 60 °C, the S48 block phase separates, and nanoparticles are not stable. By comparing the critical aggregation temperatures of knob-S48I48 and S48I48, it can be easily observed that the knob domain slightly depresses the nanoparticle assembly temperature (Table 1).

While optical density is useful to determine the temperature of assembly, dynamic light scattering is necessary to verify the size and formation of stable nanoparticles. Upon heating, both S48I48 and knob-S48I48 self-assemble into nanoparticles and this assembly was shown to be reversible upon cooling (Fig. 5(A)). When the temperature increased from 10 to 37 °C, both knob-S48I48 and S48I48 transitioned from unimers to nanoparticles, of a radius

previously shown to be nanoparticles [29]. This assembly is reversible, and the nanoparticles were disassembled into unimers when temperature decreased from 37 to 10 °C. Dreher and his coworkers [29] investigated diblock ELP that self assemble into spherical micelles by light scattering, fluorescence spectroscopy, and cryo-TEM. In our study, it was confirmed by DLS that S48I48 and knob-S48I48 self-assemble into nanoparticles with a diameter consistent with micelles as reported [29]. At physiological temperature (37 °C), the hydrodynamic radii of S48I48 and knob-S48I48 nanoparticles were 23.7 and 21.7 nm respectively. As observed using dynamic light scattering, the critical nanoparticle temperature (CNT) for knob-ELP is 19.5 °C while ELP without knob is 26.5 °C. This downward shift in CNT is consistent with that observed by optical density (Fig. 4). While the addition of knob to the ELP lowers the nanoparticle assembly temperature, it did not change the hydrodynamic radius. The control BSA exhibits a stable size around 4 nm, the same as the unimers of knob-S48I48 and S48I48, because BSA does not have any phase transition behavior. The ANOVA results (Fig. 5(B)) indicate that BSA, S48I48 and knob-S48I48 have similar sizes at 15 °C, while the particles size of S48I48 and knob-S48I48 had a significantly larger radii at 37 °C compared with BSA ($p < 0.01$).

Cleavage of knob-ELP

To determine if these ELPs can be utilized as a strategy to purify free knob, a thrombin recognition site was incorporated into the construct between knob and the ELP (Table 1). The knob-S48I48 construct was incubated with a thrombin cleavage solution, which partially cleaved the fusion peptide, as validated by a band near 20 kD (Fig. 6). Since the thrombin recognition site can be cleaved, there exists the possibility of harvesting recombinant knob from knob-ELP fusion peptides. More importantly, the molecular weight bands resulting from cleavage of knob from ELP further confirm the successful expression of the knob-ELP constructs.

Cellular uptake

Our previous study described the internalization of adenovirus 5 into lacrimal acini through a fiber or knob-dependent mechanism [5]. Other work supports the existence of a comparable endocytotic pathway capable of internalizing knob in the liver [8]. To determine if knob-mediated internalization is conferred to knob-ELP fusion peptides, live cell uptake experiments were conducted to study the internalization of knob-S48I48 into a hepatocyte cell line. This study was carried out in transformed mouse hepatocytes because of the high expression of CAR, which has been hypothesized to mediate the novel fiber and knob-dependent endocytotic uptake that has been observed. Prior to uptake studies, it was necessary to confirm that the hepatocyte cell line does express CAR (Fig. 7). A western blot comparing CAR expression in three representative and commonly utilized cell types indicated that hepatocyte lysates showed very strong immunoreactivity around 46 kD in mouse hepatocyte cell lysate only, which is the correct molecular weight for CAR [4]. CHO cell lysates showed a slight band and there was no expression detectable in Hela cell lysates.

Having demonstrated that the hepatocyte cell line expresses CAR, a rhodamine-labeled knob-S48I48 was employed to explore uptake via the CAR pathway. A rhodamine-labeled S48I48 was used as a control for cell-surface binding of ELPs. With 30 minutes incubation at 37 °C, there was a significant cellular uptake of knob-S48I48 (Fig. 8). For reference, LysoTracker green was used to stain low pH lysosomes inside the cells. A control sample without ELP shows no signal (absence of red labeling). In contrast, both knob-S48I48 and S48I48 can be clearly seen at the surface of the hepatocytes. Compared with S48I48, knob-S48I48 exhibited much stronger punctate red fluorescence inside hepatocyte cells, and S48I48 exhibited slightly more intense fluorescence on the cell surface. Both the intracellular fluorescence of knob-S48I48 and S48I48 that was seen was co-localized with

low pH compartments; however, only knob-S48I48 showed an abundant punctate intracellular fluorescence labeling pattern (Fig. 8).

Having demonstrated an effect of the fused knob domain on the cellular internalization of the fluorescent label, a competitive binding study was used to determine the specificity of uptake for the CAR pathway (Fig. 9). Pre-incubation with anti-mouse CAR antibody reduced the intracellular punctate fluorescence associated with intracellular knob-S48I48 nanoparticles relative to the signal detected in hepatocytes without antibody pre-binding. This result suggests the anti-mouse CAR antibody blocks or alters the internalization of knob-S48I48 into hepatocytes. Incubation with a non-specific antibody similarly did not affect knob-S48I48 uptake (data not shown). In conjunction with the previous experiment, this data supports a model of uptake of knob-ELP nanoparticles via a unique CAR-mediated endocytotic pathway.

Discussion

ELP fusion technology offers many advantages in protein purification [20–23] and biocompatibility [30]; however there are few reports of ELP fusion peptides as drug carriers. Floss *et al* purified an anti-HIV antibody–ELP fusion protein in transgenic plants and characterized this antibody with CHO cells, but they have not attached these fusion domains to the surface of ELP nanoparticles [31]. In contrast, Chilkoti and his group members were the first to construct diblock ELP copolymers that form nanoparticles [29]; furthermore, they modified these nanoparticles with a low affinity, low molecular weight ligand directed at traditional integrin-based targets [32]. To the best of our knowledge, our manuscript is the first description of a functional high molecular (> 10 kD) weight fusion protein (knob) located at the surface of an ELP nanoparticle. Even more surprising, the dimer and trimerization of the knob domain had almost no effect on the diameter of these polypeptide nanoparticles.

In this study we developed a simple gene product-- a diblock ELP fusion peptide that assembles nanoparticles decorated with the knob domain of the adenovirus fiber protein. Unlike low affinity targeting peptides, the knob domain is a high affinity ligand for the CAR protein. The diblock copolymer has two ELP motifs, each with distinct phase transition temperatures that result in the assembly of compact nanoparticles. The diblock ELP and diblock knob-ELP were easily purified using the ELP phase separation. SDS-PAGE indicated that the ELP and knob-ELPs were highly enriched by this purification. In comparison with traditional chromatography, the ELP phase separation is a rapid and potentially low-cost purification method.

The result of non-denaturing PAGE indicated that knob-S96, knob-I96 and knob-S48I48 were able to form dimers or trimers, which is one of the natural properties of fiber/knob protein. Most surprising, the diblock copolymer knob-S48I48 formed trimers significantly better than the monoblock ELPs knob-S96 and knob-I96. One possible explanation for this observation is that the nanoparticle surface provides a close and oriented environment, which promotes proper formation of quaternary protein structures during cellular expression.

The transition temperature results revealed that the knob-S96 and knob-I96 had one transition behavior corresponding roughly to their one ELP block. In contrast, knob-S48I48 and S48I48 demonstrated two phase transition temperatures. Between these two temperatures, dynamic light scattering indicated that both knob-S48I48 and S48I48 form nanoparticles. In addition, formation of these structures is reversible, which fits the proposed hypothesis (Fig. 1). Interestingly, the knob domain lowered the critical nanoparticle temperature compared to the plain ELP block copolymer. This type of shift in the phase

transition temperature has been reported for other fusion domains that are hydrophobic [33]. It was reported that knob has a significant number of hydrophobic amino acids [28]. Therefore, knob-S48I48 may form nanoparticles at lower temperature, while S48I48 remains unimeric. Similar results were reported by Chilkoti *et al* [32], that the diblock ELP with the low affinity ligand RGD assemble nanoparticles that range from 25 to 30 nm in radius.

The distribution of S48I48 and knob-S48I48 in cells was shown in Fig. 8. S48I48 conjugated with rhodamine exhibited a strong fluorescence signal (red) on the surface of hepatocytes, but knob-S48I48 exhibited stronger signals within the hepatocytes. From Fig. 4, both S48I48 and knob-S48I48 were shown to form nanoparticles of a similar size at physiological temperature. In the knob-S48I48 uptake experiment, most of the internalized knob-S48I48 was colocalized with the LysoTracker green, which indicates the low pH compartments. The cellular uptake studies in hepatocytes demonstrated that knob-S48I48 exhibited higher internalization efficiency into hepatocytes.

The competitive binding experiments implied that the anti-mouse CAR antibody was able to bind the CAR receptor on the cell surface of hepatocytes because of high affinity between the antibody and CAR. The pre-bound antibody blocked the internalization of knob-S48I48 and this provided evidence that the internalization of knob-S48I48 utilized the novel fiber- and knob-dependent internalization pathway previously described.

Through our generation of a novel polypeptide nanoparticle labeled with a large protein targeting ligand, this study illustrates the utility and flexibility of the ELP platform for targeting of polypeptide nanoparticles to myriad potential internalization pathways. Many limitations in drug delivery are due to a poor understanding of the unique features that exist in specific target tissues that may be used to enhance selective uptake of targeted drug delivery constructs. Our strategy for a one step genetic synthesis of a self-assembling polypeptide delivery vehicle containing a unique targeting protein may be broadly utilized as additional specific targeting pathways are identified.

Conclusion

To develop a novel targeted drug carrier, the knob domain of fiber protein from adenovirus 5 was fused with a diblock ELP capable of assembling nanoparticles. Plasmids encoding knob-ELP and ELP were constructed and purified from *E. coli*. Nondenaturing PAGE demonstrated that knob-ELP fusion peptides form trimeric and dimeric quaternary structures, which is a property of the native knob. Dynamic light scattering indicated that both knob-S48I48 and S48I48 can self-assemble into compact nanoparticles, with hydrodynamic diameters around 40 nm. The critical nanoparticle temperature of S48I48 and knob-S48I48 were 26.5 and 19.5 °C respectively. Cellular uptake experiments indicated that both S48I48 and knob-S48I48 bind a hepatocyte cell line; however, the knob-S48I48 showed more intracellular vesicular uptake, specifically into lysosomal compartments. A competitive binding experiment with anti mouse CAR antibody blocks the internalization of knob-S48I48, suggesting that uptake is mediated by knob-CAR binding and endocytosis. Unlike adenovirus, this simplified fusion peptide lacks many of the capsid proteins responsible for immunogenicity; furthermore, the knob-domain lacks the adenoviral RGD motif that targets integrins. As such, these polypeptide nanoparticles are a potentially useful new class of drug carriers that target a unique uptake mechanism, which is differentially expressed throughout the body.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Schematic representation of knob-ELP fusion peptides. Full-length knob was expressed at the N-terminus of diblock ELPs. A thrombin cleavage site was engineered between the knob domain and the diblock ELP. The designed diblock knob-ELP can assemble into a nanoparticle, mediated by the first ELP phase transition of the diblock ELP. Above the first transition temperature, knob-ELP will reversibly self-assemble into a nanoparticle.

Fig. 2. Denaturing SDS PAGE for knob-ELPs. The knob-I96, knob-S96 and knob-S48I48 were purified by inverse phase transition cycling (ITC) and resolved by SDS-PAGE stained with Coomassie brilliant blue. The molecular weights of marker proteins lane are 250, 150, 100, 75, 50, 37, 25 and 20 kD, as listed.

Fig. 3. Native-PAGE of knob-S48I48. The knob-S48I48 was mixed with non-denaturing sample buffer (without SDS or 2-mercapto-ethanol) and resolved by PAGE. The gel was then stained with Coomassie brilliant blue. The molecular weights of the marker lanes are 250, 150, 100, 75, 50, 37, 25 and 20 kD (from top to bottom), respectively.

Fig. 4. Temperature-dependence of the optical density for ELP fusion peptides. Phase transition behavior of knob-S96, S48I48 and knob-S48I48. Each sample was diluted to 25 μ M in PBS at 4 °C. The OD of samples at 350 nm was measured using a spectrophotometer with increasing temperatures from 15 to 80 °C.

Fig. 5.

Temperature-dependent assembly and disassembly for ELP fusion peptides. Dynamic light scattering was used to characterize S48I48 and knob-S48I48. (A) S48I48 and knob-S48I48 were diluted at 25 μ M in PBS and passed through a 20 nm filter at 4 °C before measurement in a DynaPro plate reader. Readings were taken starting with an increase from 10 °C to 37 °C and then a decrease from 37 °C to 10 °C. BSA was only measured from 10 °C to 37 °C. (B) Statistical comparison for nanoparticles radius for BSA, S48I48 and knob-S48I48 at 15 °C and 37 °C. *** indicates $p < 0.01$ as determined using the Tukey post-hoc test.

Fig. 6. Proteolytic release of knob from knob-ELP. Left lane is knob-S48I48 before cleavage and right lane is knob-S48I48 after incubation with thrombin overnight at room temperature. The gel was stained by Coomassie brilliant blue. The position of the cleaved knob domain near 20 kD is shown in the right lane.

Fig. 7. Transformed murine hepatocytes expresses coxsackie adenovirus receptor (CAR). Hepatocytes, CHO, and Hela were lysed with SDS-PAGE sample buffer and qualified with western blot via primary goat anti mouse CAR and mouse anti-actin antibodies, as well as secondary IRDye®800 donkey anti-goat and IRDye® 700 goat anti-mouse antibodies.

Fig. 8.

Live cell imaging of cellular uptake for ELP and knob-ELP nanoparticles. Transformed mouse hepatocytes grown on 35 mm glass-bottomed dishes were incubated in medium containing 10 μ M rhodamine-conjugated S48I48 or knob-S48I48 (red) with 75 nM LysoTracker green (green) at 37°C for 30 minutes and imaged using confocal microscopy. Knob-S48I48 exhibited markedly more co-localization with LysoTracker green while S48I48 exhibited more apparent surface association. The arrows indicated the internalized nanoparticles co-localized with lysosome. Scale bar: 10 μ m.

Fig. 9.

Competitive binding and uptake of knob-S48I48 with anti-mouse CAR antibody. Live cell imaging was performed on transformed mouse hepatocytes grown on 35 mm glass-bottomed dishes were pre-incubated with 20 $\mu\text{g}/\text{mL}$ anti-mouse CAR antibody at 37 $^{\circ}\text{C}$ for 30 minutes. Rhodamine-conjugated knob-S48I48 (red, 10 μM) with LysoTracker green (green) was added into the medium. After 30 minutes the cells were rinsed with fresh warm medium and imaged using confocal microscopy. Scale bar: 10 μm .

Table 1

Summary of Expressed polypeptides

Peptide label	* Amino acid sequence	** Critical aggregation temperature(°C)	Expected molecular weight (kD)	*** Measured molecular weight (kD)	**** Hydrodynamic radius (nm)at 37°C
knob-S96	knob-(VPGSG) ₉₆ Y	68.2	60.179	-	5.3
knob-I96	knob-(VPGIG) ₉₆ Y	-	62.682	-	-
knob-S48I48	knob-(VPGSG) ₄₈ (VPGIG) ₄₈ Y	19.5	61.431	61.241	21.7
S48I48	G(VPGSG) ₄₈ (VPGIG) ₄₈ Y	26.5	39.643	39.670	23.7

* Knob amino acid sequence with thrombin cleavage site underlined:

GATVGNKNNDKLTLWTTAPSPNCRLNAEKDAKLTLVLTKCGSQILATVSVLA VKGSLAPISGTVQS AHLIIRFDENGVLNNSFLDPEYVWNRNGDLTEGTAYTNAVGFMPNLSA YPKSHGKT
AKSNIVSQVYLNKGDKTKPVTLTITLNGTQETTPSA YSMSFSWDWSGHNYINEIFATSSYTFSYIAOEGLYVPRGSG

** determined using optical density by UV-Vis spectrophotometer.

*** determined using MALDI mass spectrometry.

**** determined using dynamic light scattering.