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Tear-mediated delivery of nanoparticles through transcytosis of the lacrimal gland

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Abstract

Rapid clearance from the tears presents a formidable obstacle to the delivery of peptide drugs to the eye surface. This impedes therapies for ocular infections, wound healing, and dry-eye disease that affect the vision of millions worldwide. To overcome this challenge, this manuscript explores a novel strategy to reach the ocular surface *via* receptor-mediated transcytosis across the lacrimal gland (LG), which produces the bulk of human tears. The LG abundantly expresses the coxsackievirus and adenovirus receptor (CAR); furthermore, we recently reported a peptide-based nanoparticle (KSI) that targets CAR on liver cells. This manuscript reports the unexpected finding that KSI both targets and transcytoses into the LG acinar lumen, which drains to tear ducts. When followed using *ex vivo* live cell imaging KSI rapidly accumulates in lumen formed by LG acinar cells. LG transduction with a Myosin Vb tail, which is dominant negative towards transcytosis, inhibits luminal accumulation. Transcytosis of KSI was confirmed *in vivo* by confocal and TEM imaging of LG tissue following administration of KSI nanoparticles. These findings suggest that it is possible to target nanomaterials to the tears by targeting certain receptors on the LG. This design strategy represents a new opportunity to overcome barriers to ocular delivery.

Keywords

Coxsackievirus and adenovirus receptor; elastin-like polypeptide; knob; transcytosis; lacrimal gland

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1. Introduction

Controlled drug delivery to the ocular surface seems intuitively straightforward, yet remains a major challenge. Many drugs are delivered as topically-added eye drops, leading to dilution of the added drug due to lacrimation, low contact time caused by rapid tear turnover, poor penetration through native barriers such as the ocular surface mucin layer, and rapid drainage of added drug through the nasolacrimal ducts [1]. Studies show that applied drug is washed away within 15–30 sec, while less than 5% of the drug administered through eye drops reaches the target tissue [2]. In addition to the natural clearance barriers limiting drug absorption, treatment of acute ocular surface disorders such as keratitis [3] as well as chronic diseases including glaucoma [4] may require addition of eye drops up to hourly, challenging patient compliance [5]. In many cases, ocular diseases such as scleritis [6] and fungal [7], bacterial and viral keratitis [8] must be treated systemically through oral or intravenous drug delivery to get enough drug to the target area, resulting in significant drug exposure at non-target sites. To overcome these challenges, various strategies, including invasive and noninvasive approaches [9, 10], have been developed to increase ocular bioavailability, improve precorneal residence time, and prolong therapeutic efficacy after topical application. The noninvasive strategies frequently focus on *in situ* gelling systems and nanoparticle technologies. Several mucoadhesive and viscosity enhancing polymers, such as polyacrylic acid-(Carbopol®) and polysaccharide-, including gellan gum (Timoptic XE®) and xanthan gum (Timolol Gel Forming Solution®), based polymers [11], have been incorporated into ophthalmic formulations now approved by the United States Food and Drug Administration. In addition, colloidal dosage forms have also been developed to increase drug stability, overcome drug efflux in conjunctival cells, and reduce dosing frequency[10]. Invasive strategies include the developments of eroding and noneroding implants, such as collagen shields [12] and pumps, have been reported to continuously deliver to the ocular surface. While promising, these approaches can compromise vision during treatment. Each strategy has its own advantages and drawbacks; furthermore, the choice of strategy depends on the envisaged therapeutic use. Thus, there is a need to explore alternative ophthalmic drug delivery strategies.

The natural source of tear fluid and proteins is the lacrimal gland (LG), an exocrine gland composed largely of acinar epithelial cells (LGAC), polarized epithelial cells that produce and secrete many of the proteins present in tears [13]. Tear protein release largely occurs from mucous and serous secretory vesicles sequestered in the acinar cells which are mobilized upon stimulation by neurotransmitters released by parasympathetic and sympathetic innervating neurons [14]. Alternatively, some tear proteins are of serum or paracrine origin and are secreted into tear fluid through a vesicular transport process called transcytosis, which involves vesicular transport through the acinar cells. There are two major transcytosis pathways, nonspecific and receptor-mediated. Nonspecific transcytosis mainly applies to abundant macromolecules in plasma [15]. Receptor-mediated transcytosis, on the other hand, is responsible for the uptake and transport of specific protein moieties and their peptide constituents across cellular barriers such as the endothelium or epithelium, and can be utilized for delivery of receptor-targeted drug molecules [16, 17].

An alternative strategy for delivery to the ocular surface might harness the body's own mechanisms in the LG for capturing tear constituents from the blood and releasing those constituents into tears *via* transcytosis. To explore this strategy, this manuscript describes genetically engineered elastin-like polypeptides (ELP) targeted to the LG via the coxsackievirus and adenovirus receptor (CAR). ELPs are composed of the repeated amino acid sequence (Val-Pro-Gly-Xaa-Gly)_n. These biocompatible and biodegradable [18] protein polymers assemble a secondary aqueous phase, known as a coacervate, above a transition temperature (T_t). This T_t can be precisely tuned by selection of the hydrophobicity of Xaa and the number of repeats, n , of the pentamer sequence. When ELPs with different T_t s are combined in the same polymer, they can assemble stable protein nanoparticles at temperatures between the T_t of the two ELPs [19]. ELPs can also be fused to targeting proteins that retain their cell-binding or drug-binding abilities [19–22]. This manuscript explores a specific ELP nanoparticle comprised of the diblock copolymer, SI, which has 48 serine (Xaa=Ser) pentamers at the amino terminus and 48 isoleucine (Xaa=Ile) pentamers at the carboxy terminus.

We have previously shown that the LG expresses CAR at one of the highest levels in the body [23]. CAR is a cell adhesion protein [24] targeted by the fiber capsid protein of adenovirus serotype 5 (Ad5). Tissues with high surface expression of CAR, including the LG and the liver, are highly transducible with Ad5, which suggests that under certain conditions CAR mediates internalization [23]. Although our group was the first to suggest this entry mechanism in the LG, endocytosis of CAR is supported by another study [25]. We and others have subsequently shown that the affinity of fiber protein for CAR can be replicated by truncations of its terminal domain, called knob. In a previous cell-culture study using only liver-derived cells, fusion of the knob domain to SI nanoparticles (KSI) conferred CAR-mediated internalization [19]. In contrast, for the first time this study demonstrates *in vivo* that KSI nanomaterials can be endocytosed into the LG and, surprisingly, that a subpopulation of these nanoparticles are efficiently transcytosed into the lumen.

2. Material and Methods

2.1. Materials and reagents

Terrific broth dry powder growth medium was from MO BIO Laboratories, Inc. (Carlsbad, CA). NHS-Rhodamine was from Thermo Fisher Scientific (Rockford, IL). Sulfo-Cy5 NHS ester was from Lumiprobe Corp. (Hallandale Beach, FL). Copper Chloride, Isopropyl-beta-D-thiogalactopyranoside, and Polyethylenimine were from Sigma-Aldrich (St. Louis, MO). The knob domain gene sequence cloned into vector pUC57 was from Integrated DNA Technologies (Coralville, IA). The pET-25b(+) vector was from Novagen (Madison, WI). LysoTracker® Red DND-99, fluorescein 10,000 MW dextran (anionic), and CellLight® RFP-Rab5a BacMam2.0 reagent were from Life Technologies (Grand Island, NY). The QIAprep Spin Miniprep Kit and QIAquick Gel Extraction Kit were from Qiagen (Valencia, CA). Matrigel™ was from Collaborative Biochemicals (Bedford, MA). Doxycycline was from Clontech (Mountain View, CA). 35 mm glass-bottomed culture dishes were from MatTek Corp. (Ashland, MA). 4–20% PAGEr Precast Gels were from Lonza (Rockland, ME). Tissue-Tek® O.C.T™ Compound was from Sakura Finetek USA (Torrance, CA).

2.2. Biosynthesis and characterization of ELPs

Recombinant plasmids encoding the ELP diblock copolymers, SI and KSI, were synthesized using plasmid recursive directional ligation [26]. The KSI protein polymer consists of a N-terminal 22nd β -repeat of the Ad5 fiber shaft (15 amino acids) which is necessary for structural folding [27], the full-length Ad5 knob domain (GenBank Number: AB361382.1), a thrombin cleavage site (Gly-Leu-Val-Pro-Arg-Gly-Ser), and a C-terminal (Val-Pro-Gly-Ser-Gly)₄₈(Val-Pro-Gly-Ile-Gly)₄₈Y (SI), in order. ELP gene construction was carried out in a pET25b(+) vector in TOP 10 competent cells followed by protein expression in the BLR (DE3) E. coli strain. E. coli encoding SI was amplified as reported previously [19]. E. coli expressing KSI was first grown in 5 ml terrific broth medium supplemented with ampicillin (100 μ g/ml) at 37 °C at 250 rpm overnight. 0.5 ml of overnight culture was then incubated in 1 L of terrific broth containing ampicillin. Isopropyl-beta-D-thiogalactopyranoside induction was initiated when the optical density (OD 600 nm) reached 0.5. KSI expression was induced by isopropyl-beta-D-thiogalactopyranoside (0.5 – 1 mM) at 25°C for 6 hrs. ELPs were purified using inverse transition cycling [28]. In general, at least five rounds of cycling were needed to obtain pure ELP samples. The purity of ELP protein polymers was assessed by SDS-PAGE using 4–20% gradient gels stained with a 10% (w/v) CuCl₂ staining solution.

2.3. Dynamic light scattering and zeta potential measurement of ELPs

Hydrodynamic diameters and polydispersity for each construct were measured using dynamic light scattering (DLS). Samples were prepared at 25 μ M in PBS and filtered through a Whatman Anotop filter with a 0.02 μ m pore size at 4 °C. 90 μ l of each sample was transferred to a pre-chilled 384 well microplate, centrifuged at 4 °C to remove air bubbles, and covered with 20 μ l of mineral oil to prevent evaporation. Samples were then measured by a Wyatt DynaPro plate reader (Santa Barbara, CA) over a range of temperature from 10 °C to 37 °C in 1 °C increments. Surface charge (zeta potential) of ELP protein polymers was determined on a Zetasizer (Malvern, Worcestershire, UK). Similarly, samples were prepared at 25 μ M in PBS and passed through a 0.02 μ m filter at 4 °C. 1 ml of each sample was applied to the disposable measurement cell. Zeta potential was estimated from the electrophoretic mobility across an applied electric current at temperatures above and below the assembly temperature for the SI and KSI nanoparticles (Supplementary Table S1).

2.4. Fluorescent labeling of ELPs

ELP samples were labeled with rhodamine (Rh) or sulfo-Cy5 using N-hydroxysuccinimide chemistry. For Rh and Cy5 conjugation of ELPs, reactions were performed in 0.1 M sodium bicarbonate solution (pH 8.3–8.5) at 4 °C for 3 hrs for KSI or overnight for SI, and the conjugated ELPs were separated by size exclusion chromatography on a PD10 desalting column (GE Healthcare, Piscataway, NJ).

2.5. Animals and Animal Procedures

Female New Zealand White rabbits weighing between 1.8 and 2.2 kg were obtained from Irish Farms (Norco, CA). Male BALB/c mice aged 12–14 weeks were purchased from Charles River Laboratories (Hollister, CA). All animal procedures were approved by the University of Southern California Institutional Animal Care and Use Committee and

followed the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, Revised 1996). For intra-lacrimal injection of SI and KSI and evaluation of fluorescence distribution by confocal fluorescence microscopy, male BALB/c mice were anesthetized with an i.p injection of xylazine 8 mg/kg and ketamine 60 mg/kg. The LG was exposed by a small incision along the axis between the lateral canthus of the eye and the ear. 5 μ l of 50 μ M Rh-labeled SI or KSI and 50 μ g of fluorescein 10k dextran were injected directly into the LG using a NanoFil syringe with a 33 gauge needle (World Precision Instruments, (Sarasota, FL). For analysis of fluorescence distribution by confocal fluorescence microscopy, the injected LGs were removed after 1 hr, placed in Tissue-Tek[®] O.C.T[™] compound, snap frozen in liquid nitrogen, cut into 10- μ m-thick sections, mounted to slides, and stored at -80 °C. For intra-lacrimal injection of SI and KSI and evaluation of nanoparticle distribution by transmission electron microscopy (TEM), male BALB/c mice were similarly anesthetized and injected with 5 μ l of 50 μ M Rh-KSI, 50 μ M Rh-SI, or 5 μ l of free rhodamine dye in PBS. The LG were removed and processed for TEM as described below.

2.6. Acinar cell isolation and primary culture

Primary acinar cells were collected from LG from female New Zealand white rabbits using previously established protocols [29]. The isolated LGACs were plated on 35 mm glass-bottomed dishes, coated with Matrigel[™] diluted 1:50 in Dulbecco's PBS, at a density of 6.0×10^7 cell/dish and cultured for 2–3 days in Peter's complete medium (PCM) before analysis. Rabbit LGACs prepared in this way reconstitute to form acinus-like structures with distinct basal-lateral and apical domains, a defined actin network enriched beneath the apical plasma membrane (AM) and produce mature secretory vesicles located in the subapical region beneath the lumena [30].

2.7. Adenovirus and baculovirus transduction and real-time fluorescence imaging

Adenoviral (Ad) constructs used in this study includes genes that are constitutively expressed (e.g. Ad mCherry-myosin Vb tail DN and Ad YFP-Rab27b) and others which are inducible upon addition of doxycycline (e.g. Ad GFP-actin and Ad mCherry-Rab3D). The inducible Ad constructs required co-transduction with the Tet-on Ad helper virus and addition of doxycycline to express regulatory proteins recognizing the reverse Tet repressor and allowing expression of the gene of interest. Transduction of LGACs with Ad constructs was done on the second day of culture. All Ad constructs were used at a multiplicity of infection of 5 and analyzed 16–18 hrs after transfection. To study the internalization of KSI, reconstituted rabbit LGACs co-transduced with Ad GFP-actin and Ad Tet-On at 37 °C were utilized to highlight the basolateral membrane (BLM) and apical membrane (AM) regions, as described previously [31]. To inhibit the transcytosis of KSI, Ad mCherry-myosin Vb tail DN was utilized, a truncated mutant of myosin Vb N'-terminally fused with a mCherry fluorescent protein tag [32]. Other constructs were also utilized to label specific intracellular trafficking pathways. To label early endosomes, LGACs were incubated with Cell Light RFP-Rab5a BacMam 2.0, a modified baculovirus expressing a fusion construct of the early endosome marker, Rab5a, and red fluorescent protein (RFP), at a final concentration of 30 particles per cell on day 2 of culture followed by 16 to 18 hrs incubation at 37 °C. The transduced cells could be identified visually by the expressed vesicular red fluorescence. For

intracellular trafficking studies, these transduced LGACs were incubated with 30 μM of Cy5-KSI at 37 °C for 60 min before imaging or, alternatively, pulsed with 30 μM of Cy5-KSI at 37 °C for 10 min. The Cy-KSI was then removed and LGACs were chased for 45 min with simultaneous imaging by confocal fluorescence microscopy utilizing a Zeiss LSM 510 Meta NLO imaging system (Thornwood, NY) equipped with Argon, red HeNe, and green HeNe laser, and a Coherent Chameleon Ti-Sapphire laser mounted on a vibration-free table.

2.8. Quantification of fluorescence signal

For evaluation of the percentage of fluorescence recovered within the cellular area versus the luminal region, the luminal and cytosolic areas within each LGAC cluster were selected by defining the regions of interest. The fluorescence intensities within these regions were analyzed using ImageJ v1.43u (US National Institutes of Health, Bethesda, MD); fluorescence intensity within each region of interest was determined by calculating the integrated fluorescence intensity corrected for background. The ratio of fluorescence intensity in the lumen to that in the cytosol was calculated. For optimal resolution, each fluorescent image was converted from an RGB to an 8-bit grayscale image before analysis. The fluorescent intensities, presented in the X-axis of Figure 2, 5 and 6 are expressed as pixels/area.

2.9. Transmission electron microscopy

The morphology of the SI and KSI nanoparticles was observed by cryogenic transmission electron microscopy (cryo-TEM). ELP solutions were kept in an ice bath (4 °C) before processing and then raised to 37 °C immediately prior to cryo-TEM sample preparation using an FEI Vitrobot. A typical procedure involves several steps as described below. In brief, ~6 μL of the sample solution was first loaded on a TEM copper grid coated with a lacey carbon film, and the grid was placed in the Vitrobot chamber with controlled temperature and humidity. After blotting of the excess solution using preset Vitrobot parameters, the grid containing a thin solution layer (~less than 300 nm) was plunged into a liquid ethane reservoir that was cooled and surrounded by liquid nitrogen. After approximately 30 sec, the sample was carefully transferred to a liquid nitrogen Dewar and stored in liquid nitrogen temperature before imaging. Throughout the imaging process, the cryo-TEM samples were kept at a temperature below -170 °C. For analysis of ELPs in vivo by transmission electron microscopy (TEM) the LG in male BALB/c mice was injected with Rh-KSI, Rh-SI, or free rhodamine dye as described above. The LG were removed and fixed in half-strength Karnovsky's fixative solution at 4 °C overnight. After fixation, the samples were carefully minced into 1-mm³ pieces, rinsed three times in 0.1M cacodylate buffer, postfixed in 2% osmium tetroxide on ice for 2 hrs, and stained en bloc with 1% uranyl acetate overnight. The samples were dehydrated in serially graded ethanol and infiltrated in eponate resin prior to embedding. The sections were cut at a thickness of 75 nm, placed on copper grids and examined at 100 kV. To compare the size of two types of nanoparticles obtained from cryo-TEM and TEM, SI and KSI were randomly selected and quantified with ImageJ.

2.10. Statistics

Values are presented as mean $\pm$ SD. Data from different experiments with only two groups were analyzed using an unpaired two-tailed student's t-test (GraphPad Prism 5.0.1). Experiments with four groups were compared with a global ANOVA followed by the Tukey post-hoc test. To satisfy the homogeneity of variance assumption, the raw intensity values were transformed by the Log₁₀ function prior to ANOVA. The criterion for statistical significance was $p < 0.05$.

3. Results and Discussion

3.1. Characterization of ELP diblock copolymer nanoparticles

The immediate goal of this study was to explore the internalization pathways of ELP nanoparticles displaying the Ad5 fiber knob domain in LGACs. SI and KSI are diblock copolymer ELPs, consisting of the N-terminal hydrophilic (Val-Pro-Gly-Ser-Gly)₄₈ moieties and C-terminal hydrophobic (Val-Pro-Gly-Ile-Gly)₄₈ moieties. Above a critical micelle temperature, both constructs assemble monodisperse nanoparticles (PDI < 0.1) with slightly negative zeta potentials (−5.9 to −7.9 mV) (Supplementary Table S1, Supplementary Figure S1A). At neutral pH the amino and carboxy termini of SI carry positive and negative charges respectively; however, nanoparticle assembly has no effect on zeta potential. As SI lacks charged amino acids, this suggests that SI nanoparticles are stabilized by steric repulsion provided by the hydrophilic ELP, and not by electrostatic repulsion. With the addition of the adenoviral knob protein, KSI showed a slightly negative shift in zeta potential, which suggests it may be stabilized by a combination of steric and electrostatic forces. Since cell surfaces are negatively charged, the stabilization of KSI may help prevent nonspecific electrostatic adsorption and promote CAR-mediated specificity. Then, SDS-PAGE was used to characterize the molecular weights of ELP constructs (Supplementary Figure S1B) revealing a molecular mass of ~38 kDa for SI and ~60 kDa for KSI, consistent with the theoretical molecular masses reported in Supplementary table 1. The image was analyzed using ImageJ to reveal the purity as 98.5% for SI and 94.5% for KSI.

3.2. Dimensions and size homogeneity of ELP nanoparticles with and without the knob domain

We next determined the size homogeneity and morphology of KSI compared to that of its SI counterpart. The hydrodynamic diameters of nanoparticles assembled by SI and KSI, determined by dynamic light scattering (DLS), were 47.1 ± 1.8 nm and 43.2 ± 0.6 nm, respectively ($p < 0.0001$) (Supplementary Table S1). In prior reports, the addition of the Ad5 knob domain reduced the critical micelle temperature slightly; however, the hydrodynamic diameter and stability at physiological temperatures was nearly unaffected by the fusion of Ad 5 knob domain [19]. Despite the increased molecular weight for monomers of KSI compared to SI, the resulting nanoparticles have nearly identical sizes. This suggests that KSI nanoparticles stabilize at a lower number of polymers per particle compared to SI. If so, this might result from a larger hydrophilic fraction for KSI (66% vs. 48% for SI), which could result in a larger radius of curvature per polymer [33, 34].

To explore any confounding difference between SI and KSI nanoparticles, cryo-TEM was employed (Figure 1A). For this analysis, particles suspended in PBS were shock-frozen in liquid ethane. The suspension was supercooled to form a vitrified thin layer so that the particles could be directly studied *in situ*. Figure 1A shows that SI and KSI form nearly spherical particles with similar size homogeneity. Particle sizes of SI and KSI, measured by DLS and cryo-TEM, are presented in Figure 1B. The diameter of the KSI nanoparticles (32.0 ± 3.5 nm) from cryo-TEM was similar to that of the SI nanoparticles (32.4 ± 4.2 nm) and there is no statistically significant difference between the two constructs (Figure 1B). The particle sizes determined by cryo-TEM are slightly smaller than the sizes reported from DLS, a difference possibly resulting from the presence of the hydration layer on the surface of core-shell ELP nanoparticles. This shell can be detected by DLS but is not visible by cryo-TEM. A similar phenomenon is observed for soft colloidal nanoparticles but not hard shell particles [35]. Moreover, the hydrodynamic diameter from DLS measures an average that is also influenced by the slight irregularity in the shape of the particles. Despite the slight differences in size, morphologies of both KSI and SI from cryo-TEM are generally consistent with the results from DLS. These observations suggest that fusion of Ad5 knob domain to the SI core nanoparticle minimally influences the nanoparticle diameter and morphology and that differences in their cellular trafficking result from receptor-mediated interactions.

3.3. Internalization and transcytosis of ELP fusions in LG acini

KSI exhibits a fiber knob-dependent and CAR-mediated endocytosis in transformed mouse hepatocytes; however, prior to this report it was unknown if these particles would interact with the LG. Similarly to hepatocytes, cells of the LG abundantly express CAR; therefore, LG CAR could be an excellent target for the selective delivery of KSI. To explore this hypothesis, three-dimensional cultures obtained from rabbit LGs were used to investigate the targeting and internalization of KSI. As documented in previous studies, isolated primary LGACs assemble into ovoid clusters after two days in culture, mimicking the acinar-like structures present in the LG [36]. A schematic of a typical reconstituted acinus comprised of LGACs is shown in Figure 2A (right) with several reference points, including a central lumen bounded by the apical membrane (AM) of adjacent cells, mature secretory vesicles (SVs) in the subapical region, beneath the basolateral membrane (BLM).

As shown in Figure 2A, fluorescently labeled Rh-SI or Rh-KSI were incubated with LGACs transduced with Ad GFP-actin. GFP-actin was used to delineate the apical lumen of reconstituted LG clusters due to its incorporation into the dense sub-apical meshwork of actin filaments proximal to the lumen. In addition to detection of intracellular puncta, presumed to be membrane compartments, Rh-KSI was clearly observed within the luminal area of LG clusters, compared with control Rh-SI. This suggests an active transport mechanism moves KSI from the basolateral to the apical membrane in these polarized LGAC cultures. The confocal fluorescence images of LGAC clusters were quantified by ImageJ to compare average fluorescent intensities in cytosol and lumen (Figure 2B) and estimate the ratio of nanoparticles between the lumen to cytosol (Figure 2C). The fluorescence intensity in the lumen of acini exposed to KSI was much higher than that in cytosol, while for acini exposed to SI, the fluorescence intensity in the lumen was lower than

that in the cytosol ($p=0.001$). When the lumen intensity was normalized by the cytosolic intensity, the ratio for KSI was significantly ($p = 0.009$) higher than for the SI control. This finding suggests that the addition of the knob domain to SI enhances the basolateral-to-apical transcytosis of the ELP nanoparticles, which possibly paves a way to utilize the KSI nanoparticle to selectively deliver therapeutic agents to lacrimal epithelial cells, tear ducts, and the ocular surface.

3.4. Characterization of KSI intracellular trafficking in LGACs

Previous reports suggested that the Ad5 knob domain administered through intravenous injection is internalized in the liver and sorted into acidic compartments of hepatocytes for degradation [37]. We hypothesized that the uptake of KSI in rabbit LGACs should involve the classical endosomal pathway and further that KSI remaining in acini that did not appear to pass to the lumen would be recovered in acidic lysosomal compartments. To evaluate this, Rh-SI or Rh-KSI was incubated with rabbit LGACs at 37 °C for 60 min before confocal fluorescence microscopy imaging (Figure 3). LysoTracker green was used as an acidic compartment marker for late endosomes and lysosomes in LGACs. As can be seen in Figure 3, LGACs without any ELP treatment showed no fluorescence signal (absence of red label). LGACs incubated with Rh-SI showed significant surface association as well as enhanced labeling of single cells present in the preparation. Some internalization was seen in puncta that colocalized with LysoTracker-labeled compartments (arrowheads). In contrast, almost no surface labeling of acini was seen for Rh-KSI which instead mainly internalized to puncta partially co-localized with LysoTracker green; furthermore, Rh-KSI again showed significant luminal Scale bars indicate 5 μ m.

Early endosomes are well-established recipients of endocytosed material in many cell types [38]. In epithelial cells internalized cargo is first trafficked into basolateral early endosomes (BEE). BEE can either be transported to common recycling endosomes and apical recycling endosomes consecutively before the release of cargo into the apical lumen, or alternatively they may be delivered to late endosomes and then lysosomes [39]. To investigate whether internalized KSI traffics to BEE in the LG, reconstituted LGACs, transduced with the BEE marker RFP-Rab5a were incubated with fluorescence labeled Cy5-KSI and analyzed by time-lapse confocal fluorescence microscopy (Figure 4 and Supplementary Video S1). Similarly to Rh-KSI, Cy5-KSI also trafficked to the lumen of LGACs, indicating that this effect is dye independent. Figure 4A shows that the Cy5-KSI is enclosed by Rab5a-enriched vesicles by 60 min, indicative of the delivery of internalized KSI into Rab5a-enriched BEEs. As seen in Figure 4B, a pulse-chase experiment with labeled KSI over a time course of 45 min of incubation showed the internalization of KSI to BEEs was observed by 10 min. In particular, the internalized KSI underwent a dynamic continuous exchange and redistribution from smaller BEEs to larger BEEs, possibly through homotypic fusion of early endosomes (Supplementary Video S1). A loss of fluorescence signal, possibly due to photobleaching or transfer of some KSI from early endosomes to proximal trafficking compartments was observed at later time points. Combined with data in Figure 3, these results suggest that KSI internalized by receptor-mediated endocytosis is first sorted into Rab5a-enriched BEEs, and then a fraction of this endocytosed material is delivered to late endosomes and lysosomes.

Having identified the primary routing steps for endocytosed KSI in LGACs, we next explored the subsequent trafficking steps involved in release of KSI into the apical lumen. Previous studies from our laboratory have identified at least three pathways for apically directed secretion of tear proteins in LGACs: the Rab11a/MyosinVb mediated transcytotic pathway [32] and the Rab3D and/or Rab27b regulated secretory pathways [40–42]. Rab3D and Rab27b are predominantly localized to distinct populations of secretory vesicles in the subapical region of LGAC that originate from the trans-Golgi network, although a subpopulation expressing both Rab proteins may exist. To investigate whether the Rab3D and Rab27b regulated secretory pathways are involved in the release of KSI into the acinar lumen of LG, LGACs were transduced with either YFP-Rab27b or mCherry-Rab3D followed by imaging of cells subjected to pulse chase uptake experiments. Figure 5C shows that the internalized Cy5-KSI (red) does not co-localize with YFP-Rab27b or mCherry-Rab3D (green), indicating that the secretion of KSI is not mediated through secretory vesicles enriched in Rab3D or 27b.

We next evaluated the role of the Rab11a/MyosinVb regulated transcytotic pathway. We have shown that expression of dominant-negative (DN) myosin-Vb tail directly impairs the movement of transcytotic vesicles and their cargo from Rab11a-enriched apical endosomes to the apical membrane, thus inhibiting transcytosis in LGACs [32]. The Rab11a-enriched apical endosome is well established in the trafficking of another transcytotic cargo receptor, the polymeric immunoglobulin A receptor, responsible for transcytosis of dimeric IgA into tears [42]. In Figure 5, LGACs were transduced with recombinant adenovirus encoding GFP-actin (green) with or without mCherry-myosin Vb tail DN (red), an N-terminal mCherry fluorescent protein fused to a truncated mutant of myosin Vb motor, which associates with vesicle cargo but does not have motor function. Transduced acini were then incubated with Cy5-KSI. As can be seen in Figure 5A, the Cy5 fluorescent signal (purple) recovered within the luminal regions surrounded by apical GFP-actin was reduced in LGACs co-transduced with Ad mCherry-myosin Vb tail DN compared to that with Ad GFP-actin alone. The fluorescent KSI signal was quantified using ImageJ in the cytosol and lumena (Figure 5B) and the ratio of luminal to cytosolic fluorescence was calculated (Figure 5C). Although the dual transduction of adenovirus slightly reduced the level of KSI internalization compared to single transduction (Figure 5B), there is a statistically significant ($p = 0.031$) reduction of the KSI signal calculated from the ratio of lumen to cytosol (Figure 5C). In LGAC in which transcytotic trafficking has been selectively impaired, Cy5-KSI accumulation in the luminal region is inhibited, demonstrating that its transport to the lumen is *via* the transcytotic pathway.

3.5. Characterization of KSI intracellular trafficking *in vivo*

To further evaluate the potential of KSI as a targeted vehicle *in vivo* our next step was to confirm the internalization and transcytotic behavior of KSI in a mouse model. First, we confirmed the expression and biodistribution of CAR in the LG of BALB/c mice by immunofluorescence, which revealed primarily basolateral enrichment with traces at the apical membrane (Supplementary Figure S2). Rh-SI or Rh-KSI, mixed with fluorescein 10k dextran as a marker of fluid phase uptake, was administered using intra-lacrimal injection. 1 hr after injection the LG was retrieved and analyzed (Figure 6A). Although fluorescein 10k

dextran is clearly present in the LG around the acinar cluster, LGs injected with Rh-SI displayed weak to no fluorescent signal in cytoplasm or lumina of LGAC, indicative of little uptake of SI *in vivo* (Figure 6A left). However, in the mice injected with Rh-KSI, KSI puncta appear inside the LGAC where fluorescein 10k dextran was recovered, suggesting that KSI was internalized. Additionally, the KSI signal was also detected in the apical region of the LGACs, suggesting that the basolateral to apical transcytosis found *in vitro* occurs *in vivo* as well. The images in Fig. 6A were quantified by ImageJ in terms of the fluorescent signals in each LG whole acinus (Figure 6B) and luminal region (Figure 6C). By considering each LG cluster, KSI displayed a 4-fold increased internalization in acini relative to SI ($p < 0.0001$) (Figure 6B). For those analyzed LG acini, the fluorescent signal in the apical region of LGACs was >7-fold higher with KSI relative to SI ($p = 0.01$) (Figure 6C). These results suggest that knob enhances the tissue affinity of SI *in vivo* and that internalized KSI can be transcytosed to the apical region of LGACs and released into the lumen.

To further confirm the internalization and intracellular trafficking of SI and KSI, we repeated the intra-lacrimal injections and analyzed the glands by TEM. The images in Figure 7 show sections from both apical and basolateral regions of mouse LG after injection with free rhodamine, Rh-SI, and Rh-KSI. The rhodamine label (not shown) was utilized to identify the area around the needle track. LGs injected with free rhodamine were used as a control group. Apical and basolateral membranes were identified based upon the relative locations of mitochondria, nuclei, secretory vesicles, and epithelial microvilli. As shown in Figure 7, no particles were observed in the control group administered with free rhodamine dye (Figure 7, free dye panels). For LGs injected with Rh-SI, SI nanoparticles could only be observed in extracellular spaces next to the basolateral membrane of mouse LG; none of these particles were seen in the apical region or lumen (Figure 7, SI panels), which is consistent with what we observed *in vitro* and with fluorescent labeling *in vivo*. However, for LGs injected with Rh-KSI, we observed internalized uniform nanoparticles enclosed in vesicular structures both at the basolateral and apical membranes of mouse LGs as well as in the lumen (Figure 7, KSI panels), again confirming the endocytosis and basolateral-to-apical transcytosis of KSI. Some KSI nanoparticles were also observed in autophagosome-like structures close to the basolateral membrane. The box-and-whisker plot shows that the diameter of SI nanoparticles (21.1 ± 4.6 nm) is similar to KSI nanoparticles (20.2 ± 2.8 nm), consistent with cryo-TEM imaging (Figure 1).

3.6. Hypothesized trafficking model of Knob-ELP

Based on the data collected previously [19] and in this manuscript, the following model is proposed for trafficking KSI in LGAC (Figure 8). Endocytosis, initiated by the binding of recombinant knob on the KSI nanoparticles to the CAR on the basolateral membrane, is followed by transport to early endosomes enriched in Rab5. Thereafter, KSI is sorted into vesicles utilizing Myosin-Vb motors for delivery to the apical membrane and released into the lumina. Prior studies have shown that transcytosis of Rab11a vesicles is Myosin-Vb dependent.[32] The remaining KSI is sorted into late endosomes, autophagosomes and lysosomes.

In this study, we observed a distinct intracellular trafficking pathway of the KSI nanoparticle in primary cells of the lacrimal gland. Prior studies of transformed hepatocytes showed that KSI nanoparticles undergo CAR-dependent internalization and are transported to lysosomes. In contrast, KSI in the LG is transported to the apical membrane and lumen through an intact transcytotic pathway. The mechanisms behind the distinct trafficking behavior in these two cell types remain to be investigated. Both hepatocytes and LGACs are specialized epithelial cells, featuring apical-basal polarity maintained by tight junctions via protein complexes, and are responsible for vectorial transport of ions and solutes across the epithelium. CAR has been identified as a regulator in tight junction permeability for many years, but its biological function and trafficking mechanism in mammalian cells remain unknown. However it is possible that a disparity in CAR function in the hepatocytes and LGACs may play a role in the different trafficking patterns of KSI-CAR complexes between hepatocytes and LGACs. CAR splice-variants have been identified in many tissues [43, 44] and each of them may exhibit alternative functions. CAR is expressed as at least two isoforms containing identical extracellular and transmembrane domains, which predict identical serotype preference and adenovirus binding, and differ only in the last 26 (CAR^{ex7}) or 13 (CAR^{ex8}) amino acids of the cytoplasmic domain [45]. CAR^{ex7} and CAR^{ex8} share a similar class of PDZ-binding domain, but interact with different PDZ-domain containing proteins, which may trigger distinct signaling and sorting pathways [46]. For example, CAR^{ex8} interaction with PDZ-domain containing protein MAGI-1b results in CAR^{ex8} degradation, while this interaction cannot be observed in CAR^{ex7}. [46] In addition, the cytoplasmic tail of CAR contains 9 lysines which may serve as ubiquitylation sites for endocytosis and trafficking of CAR to lysosomes for degradation. Deletion of this protein sequence may abolish ubiquitylation, causing resorting of CAR to the cell surface or other intracellular compartments [47].

As model systems, we investigated the intracellular trafficking and transcytosis of KSI nanoparticles in the rabbit LGAC *ex vivo* and evaluated the LG retention of KSI nanoparticles in the mouse LG *in vivo*. Part of the rationale for using two different species was that the *ex vivo* system for LGAC production requires large sources of primary tissue, which can be obtained from rabbits. In contrast, the mouse LG is easily accessible for direct injection, more so than in a rabbit. While significant anatomical diversity exists between species, many ocular elements are conserved, which suggest the translational potential of these findings. For example, rabbits have a blink rate every six min (10 sec⁻¹) [48]. Mice and rats share a similar blink rate averaged every five min each blink (12 sec⁻¹), while human has an average blink rate in every five second (0.15–0.2 sec⁻¹) [49]. These differences partially result from different eyelid configuration among species. A faster blink rate reduces the precorneal residence time on the ocular surface, increases the tear drainage, and alters the drug absorption of a topically applied therapeutics across the cornea in comparison with a slower blink rate [50]. Cornea is a transparent multilayered epithelium and serves as the primary barrier to drug absorption, especially for hydrophilic drugs. The corneal thickness varies with age, disease, external influences (e.g. contact lenses), damage, and species. Recent research has reported that transporters expressed in the corneal epithelium may involve the transport of some hydrophilic drugs [51]; however, corneal transporter-mediated uptake and elimination can vary largely between species. While

parameters such as these clearly suggest major differences between species in ocular pharmacokinetics, the KSI targets the CAR receptor, which is expressed constitutively in the LG across rabbits, mice, and humans [23]. Thus the fact that KSI transcytoses through the LGAC in two different species, suggests the possibility that it may also have this ability in the human LG.

Two different fluorescent dyes (rhodamine, Cy5) were evaluated as labels for KSI in this study. The criteria for fluorescent dye selection were based on two factors: i) the need to label KSI with either red or far red wavelength emissions compatible with other double and triple label microscopy studies; and ii) to demonstrate that KSI transcytosis was conserved across at least two distinct fluorescent probes. Both rhodamine (e.g. TRAMA) and Cyanine dyes (e.g. Cy5) are small molecule derivatives that link to primary amino acids. Rhodamine derivatives have high photostability and little sensitivity in physiological pH range and are suitable for multicolor labeling experiments [52]. In comparison, sulfo-Cy5 shows comparable photostability, more intense emission, and increased water solubility. Therefore, sulfo-Cy5 is well suited for the labeling of proteins that easily denature in the presence of organic co-solvents sometimes used for labeling reactions. Rhodamine is excited by green light; however, Cy5 derivatives are excited by red light (650nm) and emit in the far-red region (680nm). Cy5 is well suited for triple label live-cell microscopy experiments alongside probes (RFP-Rab5a, mCherry Rab3D, YFP-Rab27b, mCherry Myosin Vb tail) with emissions overlapping that of rhodamine. As KSI conjugated with either rhodamine or sulfo-Cy5 displayed comparable internalization and luminal accumulation in rabbit LGACs, this further supports the contention that KSI mediates transport across the polarized cells of the LG.

Herein, we report for the first time a protein polymer nanoparticle that selectively enters LGAC *in vitro* and *in vivo* and undergoes a basolateral-to-apical transport for secretion into the apical lumen of LGAC. LGAC lumina drain into lacrimal ducts that delivery tear proteins and electrolytes to the surface of the eye. This unusual transcytosing property of the nanoparticle provides a unique capability that may be exploited for sustained delivery of drugs to the ocular surface for those diseases that are currently difficult to treat and require continuous infusion of drug. Transcytosing nanoparticles could be developed for delivery *i.v.* or *s.c.* or alternatively injected to form a depot in case of acute infection or trauma.

4. Conclusion

As a model for targeted transcytosis to the tear ducts, this strategy has the potential to generate new nanomaterials that act at the LG, the ducts, or the ocular surface. Based on the ELP diblock copolymer template, KSI fusion proteins assemble into stable, monodisperse, and biodegradable nanoparticles at physiological temperature. Genetic fusion of the Ad5 knob domain to SI minimally affects the morphology of the particle but significantly enhances its internalization efficiency. With efficient targeting to CAR on cells of the LG, KSI nanoparticles are internalized and transported from basolateral to apical membranes. KSI nanoparticles represent the first engineered delivery vehicle with potential for selective delivery of therapeutic agents from serum, through the lacrimal gland acinar cells, and to the tears bathing the ocular surface.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ad5	adenovirus serotype 5
Ad	adenovirus
AM	apical membrane
BEE	basolateral early endosomes
BLM	basolateral membrane
CAR	coxsackievirus and adenovirus receptor
cryo-TEM	cryogenic transmission electron microscopy
DLS	dynamic light scattering
DN	dominant negative
EE	early endosome
ELP	elastin-like polypeptide
GFP	green fluorescent protein
KSI	knob fusion to an ELP diblock copolymer
LG	lacrimal gland
LGAC	lacrimal gland acinar cell
PCM	Peter's complete medium
RFP	red fluorescent protein
Rh	rhodamine
SI	ELP diblock copolymer
SV	secretory vesicle
TEM	transmission electron microscopy
T_t	transition temperature

YFP yellow fluorescent protein

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Highlights

- Genetic fusion of the Ad5 knob domain to SI enhances the internalization of KSI in LGACs.
- KSI nanoparticles are internalized and transcytosed in LGACs *in vitro* and *in vivo*.
- Internalized KSI is trafficked to early endosomes and then sub-apical compartments of LGACs for secretion.

Figure 1.

Genetic fusion of the Ad5 knob domain to SI has minimal impact on the morphology of ELP nanoparticles. (A) Cryo-TEM micrographs of ELP nanoparticles with and without the Ad5 knob domain. (B) A comparison of diameters from ELP nanoparticles imaged by cryo-TEM and DLS. For cryo-TEM, the average diameters of SI and KSI, measured with ImageJ, were 32.4 ± 4.2 nm and 32.0 ± 3.5 nm, respectively. Values are expressed as mean $\pm$ SD (n=15). For DLS, the average diameters of SI and KSI were 47.1 ± 1.8 nm and 43.2 ± 0.6 nm, respectively. Values are expressed as mean $\pm$ SD (n=10). p value < 0.0001 (Student t-test).

Statistical comparison was not performed between cryo-TEM and DLS techniques because they measure different aspects of particle formation.

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Figure 2.

KSI is internalized and accumulates in the cytosol and lumina of rabbit LGAC expressing GFP-actin. (A) Live cell imaging comparing the intracellular distribution of ELPs with and without Ad5 knob after 1 hr incubation at 37 °C. Green, GFP-actin; Red, Rh-conjugated SI or KSI; *, luminal space; Scale bar indicates 10 μ m. Schematic diagram on the top right depicts reconstituted rabbit LGACs shown on the first row (Control), and indicates the presence of apical plasma membrane (AM), secretory vesicles (SV) and the basolateral membrane (BLM). *, acinar lumen. (B) Quantification of fluorescent intensity in LGACs treated with SI and KSI. ***, p value<0.005 (ANOVA followed by Tukey post-hoc test). (C) Quantification of the fluorescence intensity expressed as a ratio of luminal to cytosolic fluorescence. **, p value=0.009 (Student T-test). For (B) (C), fluorescence intensity was analyzed using ImageJ. Data are presented as mean $\pm$ SD (n=5).

Figure 3.

ELP nanoparticles traffic to low pH compartments in rabbit LGACs. 30 μM of Rh-coupled SI or KSI were incubated with rabbit LGACs at 37 $^{\circ}\text{C}$ for 1 hr, and imaged using confocal fluorescence microscopy. KSI (red) exhibited significant co-localization with acidic compartments labelled by LysoTracker green (green) as well as luminal (*) accumulation. SI showed significant surface binding and also some internalization to acidic compartments labelled by LysoTracker green. Arrowheads indicate the co-localization of SI or KSI with acidic compartments. White lines delineate the BLM of LG acinar clusters. *, lumina. Scale bar indicates 10 μm . accumulation. Based on these findings, we propose that the internalized SI and KSI are partially transported to late endosomes and lysosomes, but that KSI internalization occurs more efficiently and is further transported *via* transcytosis.

Figure 4.

Intracellular trafficking of KSI nanoparticles in LGACs. (A) Internalization of KSI to early endosomes in live rabbit LGACs expressing RFP-Rab5a, a marker of early endosomes. LGACs transduced with RFP-Rab5a (green) were treated with 30 μ M of Cy5-KSI (red) at 37 $^{\circ}$ C for 1 hr, washed with DPBS twice, and analyzed by confocal fluorescence microscopy. Arrowheads indicate Cy5-KSI in early endosomes. (B) LGACs transduced with RFP-Rab5a (green) were pulsed with 30 μ M of Cy5-KSI (red) at 37 $^{\circ}$ C for 10 min, rinsed and followed by a 45 min chase in fresh PCM at 37 $^{\circ}$ C. The yellow-boxed region is expanded in time-lapse images shown successively to the right. Arrowheads indicate early endosomes that include Cy5-KSI at earlier times but show label dissipating at later times of incubation. White lines depict the periphery of the reconstituted cluster of LGAC obtained

by phase contrast imaging. (C) LGACs expressing mCherry-Rab3D (red) or YFP-Rab27b (red) were pulsed with 30 μ M of Cy5-KSI (green) at 37 °C for 10 min and imaged for 45 min in fresh PCM at 37 °C. Internalized KSI did not associate with either Rab3D or Rab27b in live rabbit LGACs. Data were presented at the indicated time points. White lines depict the periphery of the reconstituted cluster of LGACs. Arrowheads indicate internalized Cy5-KSI.

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Figure 5.

Overexpression of a dominant negative Myosin Vb tail impairs basolateral-to-apical transcytosis of KSI in LGACs. (A) Reconstituted rabbit LGACs, co-transduced with AdGFP-actin (green) to delineate morphology, Ad mCherry Myosin Vb tail DN (red), and Ad helper virus, were incubated with 30 μ M of Cy5-KSI nanoparticles (purple) at 37 $^{\circ}$ C for 1 hr before analysis. *, luminal region of LGACs. Scale bar indicates 10 μ m. (B) Quantification of fluorescence intensity in lumen and cytosol of LGACs with or without the dominant negative myosin Vb tail, which inhibits transcytosis. ***, p value<0.005 (ANOVA followed by Tukey post-hoc test). Data were expressed as Mean $\pm$ SD (n=4). (C) Quantification of fluorescence intensity as a ratio of luminal to cytosolic fluorescence. Data were expressed as Mean $\pm$ SD (n=4). KSI, 0.83 $\pm$ 0.48; KSI with inhibition, 0.13 $\pm$ 0.09; p value = 0.031 (Student T-test).

Figure 6.

Comparison of *in vivo* ELP nanoparticle accumulation in LG from BALB/c mice. (A) Intra-lacrimal gland injection of Rh-coupled KSI (red) into LG of 12 week BALB/c mice showed significant internalization, LG retention, and luminal accumulation compared to SI (red). Fluorescein 10k dextran (green) was used as a control and fluid-phase marker. White arrowheads indicate internalized KSI close to or in the apical/luminal region of mouse LGACs. Scale bar indicates 10 μ m. (B) Quantification of fluorescence intensity for internalized SI and KSI. The fluorescent signals were analyzed in LG acinar clusters and analyzed using ImageJ. ****, p value < 0.0001 (Student T-test). Bar are expressed as mean $\pm$ SD ($n=5$). (C) Quantification of fluorescence intensity in the subapical membrane and luminal region of clusters of mouse LGAC. Data were expressed as mean $\pm$ SD ($n=5$). *, p value = 0.01 (Student T-test).

Figure 7.

Transmission electron microscopy confirms KSI transectosis in LGs from BALB/c mice. Transmission electron microscopy images compared mouse LGs from 12 week BALB/c mice administered with free rhodamine dye, SI or KSI by intra-lacrimar injection. Vesicle-enclosed black puncta close to the apical membrane (AM) of mouse LG acini are KSI nanoparticles. L, lumen; SV, secretory vesicles; M, epithelial microvilli projecting from the AM of LG acini. Scale bars indicate 1 μ m. KSI diameters were summarized in a box-and-whisker plot. Particle diameters are expressed as mean $\pm$ SD. n = 178 and 205 for SI and

KSI, respectively. For the box-and-whisker plot, the box expresses mean $\pm$ SD and the whisker shows minimum and maximum values.

Figure 8.

Working model for intracellular trafficking of KSI ELPs in LGACs. KSI is internalized *via* CAR-mediated endocytosis. KSI is then delivered to Rab5a-early endosomes where some KSI nanoparticles are transported to late endosomes and lysosomes, whereas others are sorted to Rab11a-associated sub-apical intracellular compartments for release to the apical lumen of LG acini. Some KSI remains in intracellular acidic compartments without entering the transcytotic pathway.