Clathrin and caveolin-1 expression in primary pigmented rabbit conjunctival epithelial cells: Role in PLGA nanoparticle endocytosis

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Purpose: The internalization of poly (dl-lactide-co-glycolide, PLGA) nanoparticles in rabbit conjunctival epithelial cells (RCEC) was previously shown to occur by an endocytic process, as evidenced its energy-dependence, inhibition by the vesicle formation blocker cytochalasin D, and by the characteristic display of punctate distribution under confocal microscopy. In addition, clathrin protein was implicated in the endocytosis of these nanoparticles in vascular smooth muscle cells. We sought to examine the expression of clathrin and caveolin-1 in RCECs and to determine whether they play a role in PLGA nanoparticle endocytosis.

Methods: PLGA (50:50) nanoparticles (100 nm in diameter) containing 6-coumarin (fluorescent marker, 0.05% w/v) were used in this study. The effect of pharmacological treatments aimed at disrupting formation of clathrin-coated vesicles (hypertonic challenge and intracellular K+ depletion) and caveolae (nystatin and filipin) on apical uptake of nanoparticles in primary cultured RCEC was investigated. Transferrin was chosen as a marker for clathrin-dependent endocytosis from the basolateral aspect, whereas cholera toxin B subunit was chosen as a marker for caveolae-mediated endocytosis. The staining pattern of nanoparticles in RCECs was compared with that of clathrin heavy chain (HC) and caveolin-1 under fluorescent confocal microscopy to examine possible colocalization using clathrin HC and caveolin-1 mouse monoclonal antibodies (mAb). Two pairs of primers were designed (based on conserved regions of clathrin and caveolin-1 gene in different species) to amplify a 744-bp and 152-bp fragment of clathrin HC and caveolin-1 gene, respectively. Reverse transcription-polymerase chain reaction (RT-PCR) to detect the message for clathrin HC and caveolin-1 gene in various species was performed using total RNA prepared from freshly isolated RCECs. HEK293 cells were used as positive control for clathrin gene expression, whereas rabbit heart muscle and HEK cells were used as positive control for caveolin-1 gene expression. The RT-PCR products were separated using 2% agarose gel electrophoresis. Western blot analysis was performed to detect the expression of both clathrin and caveolin-1 in RCECs as indicated by a 152-bp fragment of the gene. Western blot analysis revealed a clathrin HC band (180 kDa) in RCEC culture and HeLa cells and A431 epidermoid cells were used as positive controls. The effect of transfection of RCECs (using Lipofectamine 2000™ reagent) with specific antisense oligonucleotides designed against the rabbit clathrin isoform on clathrin protein expression and PLGA nanoparticle uptake was investigated.

Results: Apical uptake of nanoparticles in primary cultured RCECs was decreased by 45% and 35%, respectively, as a result of K+ depletion and hypertonic media treatments. Likewise, the same treatments significantly decreased the basolateral uptake of FITC-transferrin by 50%. In contrast, nystatin and filipin had no effect on apical uptake of nanoparticles and cholera toxin B subunit in RCECs, suggesting a lack of the involvement of caveolae in the internalization of these two agents. Confocal microscopy showed fluorescent staining of cell membrane in the presence clathrin mAb, but not in the presence of caveolin-1 mAb, with partial overlap with a nanoparticle staining pattern. RT-PCR confirmed the presence of the clathrin HC gene, but not the caveolin-1 gene, in RCECs as indicated by a 744-bp fragment of the gene. However, caveolin-1 gene was detected in other rabbit tissues such as the epithelium of the cornea and trachea, and heart muscle, as indicated by a 152-bp fragment of the gene. Western blot analysis revealed a clathrin HC band (180 kDa) in RCEC culture and HeLa cells. However, caveolin-1 protein (22 kDa) was not detected in RCEC culture, but was detected in A431 cells. Transfection of RCECs with antisense oligonucleotide directed against clathrin HC resulted in knockdown of the clathrin HC protein in a concentration dependent manner. However, clathrin HC protein knockdown had no effect on apical uptake of nanoparticles in RCECs.

Conclusions: Our findings indicate that endocytosis of nanoparticles in primary cultured RCECs occurs mostly independently of clathrin- and caveolin-1-mediated pathways. In addition, the gene and protein expression of clathrin HC, but not caveolin-1, was identified in rabbit conjunctival epithelial cells.

Topical delivery of drugs by the ophthalmic route is hindered by poor absorption coupled with rapid elimination, resulting in only 1-3% of the active ingredient being taken up. The use of polymeric nanoparticles in the eye has gained considerable interest recently due to their stability, longer half-life for elimination, and a wide range of application for the
delivery of many drugs used to treat common ocular disorders. Nanoparticles have been utilized to enhance the absorption of therapeutic drugs and peptides [1,2], reduce systemic side effects [3], and sustain drug levels in the systemic circulation [4,5]. PLGA is a copolymer of polylactide and polyglycolide and is an ideal candidate of biodegradable polymers for formulation into nanoparticles due to its wide medical use, biocompatibility, and safety [6].

Endocytosis is known to account for the epithelial transport of some nutrients and most proteins and participate in the regulation of signaling receptors on the cell surface. Macromolecules are engulfed in membrane invaginations and internalized as vesicles with subsequent intracellular sorting during endocytosis. Viruses such as simian virus 40 and adenovirus-associated virus type 5 also utilize this same endocytic machinery to gain entry into the cell during infection [7,8]. Cellular mechanisms of the endocytic machinery remain under heavy scrutiny and the selectivity of the process towards certain molecules remains unclear. We have previously shown that PLGA nanoparticles loaded with fluorescent dye were internalized by an endocytic process in rabbit conjunctival epithelial cells (unpublished data). Internalization appeared to be energy-dependent, inhibitable by vesicle formation inhibitor cytochalasin D, and displayed a punctate distribution pattern under confocal microscopy following uptake. However, the molecular mechanisms of uptake of PLGA nanoparticles have not been clearly elucidated to date.

Until recently, endocytosis of substrates in mammalian cells was thought to mostly occur by clathrin-coated pits for cargo recruitment. Other endocytic pathways mediated by caveolae, (50-80 nm), macropinosomes (500-2000 nm), or micropinosomes (95-100 nm) are less well characterized [9]. These pathways do not use conventional coat complexes, instead they rely on membrane heterogeneity in composition or the presence of microdomains for cargo selection and vesicle budding [10]. Caveolae are smooth invaginations of the plasma membrane that were first described in endothelial cells [11]. They represent glycolipid- and cholesterol-rich membrane domains which are specifically associated with caveolin proteins [12]. Ligands known to be endocytosed by caveolae pathway include cholester toxin B subunit, endothelin, and IL-2 receptor [13-15].

Both clathrin and caveolin are thought to be ubiquitous proteins, although their existence and role in conjunctival epithelial cells have not been studied systematically. The localization of caveolin at the cell membrane of corneal epithelium was previously reported in the context of wound healing [16]. In addition, the presence of both clathrin and caveolin in the retina and lens was described [17-19]. Elucidation of the endocytic pathway (clathrin and/or caveolae-mediated or non-coated vesicles) involved in nanoparticle uptake is pivotal for further studies aimed at regulation of that pathway to enhance total nanoparticle uptake in these cells and to manipulate intracellular sorting and trafficking of these nanoparticles for specific targeting and avoidance of the degradative machinery.

In this study, we examined the expression of clathrin heavy chain and caveolin-1 in rabbit conjunctival epithelial cells and investigated whether they have any role in PLGA nanoparticle endocytosis using pharmacological treatments aimed at disrupting the formation of clathrin-coated pits and caveolae. We also sought to evaluate the effect of transfection with specific antisense oligonucleotides designed against the rabbit clathrin isoform on nanoparticle uptake.

**METHODS**

**Chemicals and reagents:** FITC-transferrin was purchased from Molecular Probes (Eugene, OR). Nystatin, choleicerin toxin B subunit, and filipin were purchased from Sigma Chemical Co. (St. Louis, MO). Mouse monoclonal antibodies against either clathrin heavy chain (HC) or caveolin-1 were purchased from BD Biosciences (Lexington, KY). Anti-sense oligonucleotides targeted against clathrin heavy chain and caveolin-1 genes and Lipofectamine 2000 reagent were obtained from Invitrogen Corporation (Carlsbad, CA). HeLa (human adenocarcinoma) and A431 (human epidermoid carcinoma) cell lines were obtained from ATCC (Manassas, VA). Nanoparticles of polylactic polyglycolic acid co-polymer (PLGA 50:50, inherent viscosity 1.31 measured in hexafluoroisopropanol) of mean diameter 100 nm containing 6-coumarin (0.05% w/v) as a fluorescent marker were obtained from Dr. Labhasetwar’s laboratory (Nebraska College of Pharmacy) and characterized with the methods reported by Davda and Labhasetwar [20]. Culture media was purchased from Biowhittaker (Walkersville, MD).

**Animals and tissue preparation:** Conjunctival tissue was isolated and prepared as described previously by Kompella et al. [21]. Briefly, male Dutch-belted pigmented rabbits, weighing 2.0-2.5 kg, were obtained from Irish Farms (Norco, CA) and handled in accordance with Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23). Rabbits were euthanized by an overdose injection of sodium pentobarbital solution (325 mg/kg) into the marginal ear vein. Conjunctival tissues from the excised eyeballs were carefully dissected and isolated for primary cell culture.

**Primary cell culture:** Rabbit conjunctival epithelial cells (RCECs) were harvested using a protocol developed by Saha et al. [22] and modified by Yang et al. [23]. Isolated cells were seeded at a density of 1.2x10^6 cells/cm² on ClearwellsTM (12 mm diameter, 0.4 µm pore size, Costar, Cambridge, MA) precoated with rat tail collagen and cultured in 5% CO₂ and 95% air at 37 °C. From day 4 onwards, cells were grown to an air interface condition and used for uptake studies on day 7-9 upon confluence, as judged by transepithelial resistance (TEER) and potential difference (PD) values measured using a Voltohmeter electrode (EVOM, World Precision Instruments, Sarasota, FL). Confocal microscopy studies were done on sub-confluent RCECs (80% confluent, on day 5-6), due to ease of visualization.

**Nanoparticle uptake study in RCECs and analytical methods:** The detailed uptake of nanoparticles and analysis method were described previously [20]. Briefly, following confluence of cultured RCECs, the culture medium on both sides of the
cells was replaced with a physiological bicarbonate Ringer’s solution (BRS) containing 1.8 mM CaCl₂, 5.6 mM KCl, 0.8 mM MgSO₄, 0.8 mM Na₂HPO₄, 116 mM NaCl, 25 mM NaHCO₃, 15 mM HEPES, and 5.5 mM D-glucose and incubated for 30 min at 37 °C. The BRS was bubbled with air containing 5% CO₂ and adjusted to pH 7.4 before usage. The osmolarity of the solution was in the range of 290-310 mOsm. All nanoparticle suspensions were prepared in BRS solution. After 30 min incubation with BRS, the apical side of conjunctival epithelial cells was replaced with the nanoparticle suspension and incubated for 30 min at 37 °C. The apical solution was then aspirated and the cell filters were washed three times with ice-cold BRS buffer solution to remove nanoparticles not internalized. The cell filters were then cut off from the Transwell using a blade and transferred separately into covered disposable tubes filled with 1 ml BRS solution. For the determination of the amount of membrane-bound fraction of nanoparticles, washed cells were trypsinized with 10X trypsin-EDTA solution (Invitrogen Corporation, Carlsbad, CA) for 30 min to a total volume of 1 ml and centrifuged twice at 1000x g for 10 min each. The resulting pellet was solubilized with 0.5 ml of 0.5% Triton-X 100 in BRS for 30 min at 37 °C and diluted to 1 ml with BRS. The filter samples and/or both the supernatant and dissolved pellet (1 ml each) of each sample were then frozen at -20 °C and lyophilized overnight. Extraction of coumarin from nanoparticles was done by incubating the lyophilized samples with 1 ml methanol at 37 °C for 24 h under gentle agitation. The samples were then centrifuged at 1000x g for 10 min and the supernatant collected and analyzed using a spectrofluorometer F-2000 (Hitachi, Tokyo, Japan) set at an excitation wavelength of 450 nm and emission wavelength of 490 nm. Standard curves for each nanoparticle experiment were obtained by spiking different concentrations of nanoparticles (12.5-200 µg/ml) in BRS and treated the same way as the nanoparticle samples from the experiments. Uptake data are presented as mean±standard error of the mean (n), where n is the number of observations. Both Student’s t-test and one-way and two-way ANOVA analysis (using the Tukey-Kramer test) were utilized to evaluate significant differences (p<0.05) in sample means, as appropriate.

Inhibition of clathrin- and caveolin-mediated endocytosis: Hypertonic challenge or intracellular K⁺ depletion were performed according to Hansen et al. [24] to disrupt clathrin-mediated endocytosis. Briefly, hypertonic challenge was carried out by incubating RCECs with BRS supplemented with 0.45 M sucrose for 15-20 min at 37 °C from both sides. Depletion of cytosolic K⁺ was achieved by incubating primary cultured RCECs with hypotonic BRS medium (50%) containing ouabain (1 mM) for 5 min followed by ice-cold K⁺-free BRS medium for 20 min on both sides. Once treatment was complete, RCECs were then incubated from the apical side with either PLGA nanoparticles (0.5 mg/ml) or FITC-transferrin (50 µg/ml, also used for basolateral uptake studies) for 30 min, washed three times with ice-cold BRS, and analyzed for fluorescence as described above. Treatments aimed at inhibiting caveolae-mediated endocytosis were evaluated by incubating

Figure 1. Effect of pharmacological treatments on internalization of PLGA nanoparticles, transferrin and cholera toxin B subunit in RCECs. A: Apical uptake of coumarin-loaded PLGA nanoparticles (0.5 mg/ml) in RCECs for 30 min at 37 °C under various treatments aimed at inhibiting endocytosis mediated by clathrin (K⁺ depletion and hypertonic challenge) or caveolae (nystatin and filipin). Membrane bound fractions were determined by centrifugation of the trypsinized cells 2 times following nanoparticle uptake and estimating the coumarin content in the solubilized pellet fraction. The asterisk denotes significant differences from both basolateral and apical uptake (p<0.05). The plus sign denotes significant differences from apical uptake (p<0.05). Bars represent mean±standard error of the mean (n=6).
RCECs from both sides with a sterol-binding agent, nystatin or filipin at 5 µg/ml, for 30 min at 37 °C followed by apical uptake of nanoparticle (0.5 mg/ml) or FITC-cholera toxin B subunit (5 µg/ml), a substrate known to be internalized by caveolae-mediated endocytosis in other cell systems.

**Immunofluorescence and confocal microscopy:** Sub-confluent RCECs (80% confluence) were incubated from the apical side with 0.5 mg/ml suspension of PLGA nanoparticles loaded with 6-coumarin at 37 °C, and then washed three times with ice-cold BRS buffer. Cells were then fixed with 4% paraformaldehyde in PBS solution for 30 min, permeabilized using 0.5% Triton-X 100 in water for 15 min, blocked with 10% bovine serum albumin (BSA) in PBS solution for 30 min, and incubated with mouse monoclonal antibody (BD Biosciences, Lexington, KY) against either clathrin HC or caveolin-1 for 2 h. Cells were then washed several times with PBS and incubated for 1 h with rhodamine-labeled goat anti-mouse secondary antibody. Finally, the cell filter was cut and mounted on a glass slide using a Prolong™ anti-fade mounting kit (Molecular Probes, Eugene, OR) and viewed under a confocal microscope (Ziess LSM 510, Germany) using both FITC (wavelength 450-490 nm) and rhodamine filters (wavelength 550-570 nm).

**RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR):** Freshly isolated rabbit conjunctival epithelial cells were treated with 1 ml TRIzol® Reagent (Invitrogen Corp., Carlsbad, CA) and total RNA was obtained according to the manufacturer’s direction. Isolated conjunctival RNA was reverse-transcribed to cDNA using oligo-dT primers (20 bp long, SuperScript™ II, Invitrogen Corp., Carlsbad, CA). PCR was performed using sense (5'-CGG TTG CTC TTG TTA CGG-3) and antisense (5'-AGA GCA TTA AAT TTC CGG GC-3) primers based on conserved regions of clathrin HC genes cloned from both human (NM_004859) and rat (J03583). The sense primers chosen correspond to 525-542 bp of human and 451-468 bp of the rat clathrin HC gene, whereas the antisense primers correspond to 1265-1284 bp of human and 1191-1210 bp of rat clathrin HC gene. For caveolin-1, PCR was performed using sense (5'-CAA CTA CAA GCC CA-3) and antisense (5'-AAA CTT CTA CAC TAA CG-3) primers based on conserved regions of the caveolin-1 gene cloned from human (Z18951), dog (U47060), rat (Z46614), mouse (U07645), and chicken (L01582). The sense primers correspond to 344-360 bp of the human, 134-150 bp of the dog, 94-110 bp of the rat, 66-82 bp of the mouse, and 135-151 bp of the chicken caveolin-1 gene. The antisense primers cor-

Figure 2. Confocal microscopy of RCECs following uptake of PLGA nanoparticles. A and B represent x-y confocal images (40x magnification) scanned below the apical layers of sub-confluent RCECs (80% confluence). A: RCECs stained with clathrin HC antibody (red). B: Uptake of PLGA nanoparticles containing coumarin (green) after incubation for 30 min at 37 °C. C: Phase contrast image of RCECs. D: Merged image of A and B showing partial colocalization of the staining of coumarin-nanoparticles with that of clathrin HC. Areas of overlap are denoted by arrowheads. These findings were confirmed with magnification of up to 100x, however, for best illustrative quality without compromising image resolution only the 40x magnified specimens are shown.
respond to 480-496 bp of human, 270-186 bp of dog, 230-246 bp of rat, 202-218 bp of mouse, and 271-287 bp of chicken caveolin-1 gene. PCR conditions for clathrin were as follows: 94 °C for 30 s, 51 °C for 30 s, and 70 °C for 30 s, all 30 cycles and PCR conditions for caveolin-1: 94 °C for 30 s, 42 °C for 30 s, and 70 °C for 30 s, for 30 cycles (in some instances 50 cycles were performed to check for the caveolin-1 gene fragment).

The RT-PCR products were resolved under agarose gel electrophoresis and cDNA amplicons corresponding to predicted product sizes of clathrin HC purified using the QIAquick Gel Extraction kit (Qiagen Inc., Valencia, CA). The resultant cDNA fragments were ligated into a pGem®-T Easy vector (Promega Corp., Madison, WI) and the vectors were expanded in competent cells. Positive clones were selected and analyzed by endonuclease (EcoR I) restriction assay. The resultant plasmid was then sequenced using infrared fluorescent dye-labeled M13 primers (GeneMed Synthesis Inc., San Francisco, CA). The homology of amplified fragments was verified by comparison to known clathrin HC gene sequences using standard multialign program (Clustal W sequence alignment program).

Western analysis of clathrin HC and caveolin-1: Western blot analysis was performed on cell lysates of confluent RCECs on day 7-9 using a mouse monoclonal antibody against either clathrin HC or caveolin-1. RCECs were incubated for 30 min at 37 °C with lysis buffer (1% SDS in PBS) containing 1% protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO) and 20 μl of cell lysate was used to determine the protein content using the DC protein assay kit (Bio-Rad, Hercules, CA). Cell lysates of HeLa cells were used as a positive control for clathrin HC expression, whereas A431 cells was used as a positive control for caveolin-1 expression. Rabbit cardiac muscle tissue was used as a control to demonstrate cross reactivity of mouse monoclonal caveolin-1 antibody for rabbit protein. About 50 μg of protein from each cell type (RCEC, cardiac muscle, HeLa, and A431) was then electrophoresed on 8% SDS-PAGE, followed by electrotransfer to a nitrocellulose membrane. The immoblot procedure utilizing the enhanced chemiluminescence method (ECL) was performed according to the manufacturer’s protocol (Pierce, Rockford, IL).

Treatment of RCEC with antisense oligonucleotides directed against clathrin HC gene: RCECs grown on 12 mm coated Clearwells on day 7-8 of culture with TEER values of 1.1±0.15 Ω/cm² were treated for 4-6 h with a 15-bp antisense oligonucleotide (5′-CTT CCG TCA CCT ACA-3′) directed against the rabbit clathrin HC mRNA (corresponding to fragment between 290-304 bp). The 15-mer antisense oligonucleotide was complexed with Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen Corp., Carlsbad, CA) prior to use. HeLa cells were used as a positive control for transfection. After 24 h of transfection, cell lysates were processed for western blot analysis as described above to confirm the reduction of clathrin HC protein.

RESULTS

Inhibition of clathrin- and caveolin-mediated endocytosis: As shown in Figure 1A, apical uptake of PLGA nanoparticles in RCECs decreased by 45% and 35%, respectively, as a result of intracellular K⁺ depletion or hypertonic treatment of cells for 30 min. However, 30 min pretreatment of RCECs with either nystatin or filipin, maneuvers known to interfere with caveolin-dependent endocytosis in other cells, did not affect apical uptake of nanoparticles. As a control for clathrin-mediated endocytosis, we also investigated the apical and basolateral uptake of FITC-transferrin in RCECs. Figure 1C shows that both apical and basolateral uptake of FITC-transferrin was decreased significantly by 22% and 47%, respectively, as a result of intracellular K⁺ depletion or hypertonic media treatment of RCECs. Furthermore, both the apical and basolateral uptake of FITC-transferrin was not affected with pretreatment of cells using nystatin, suggesting endocytosis of transferrin into RCECs does not occur via caveolae-dependent mechanisms. To further evaluate the role of caveolae in the endocytosis of molecules in RCECs we studied the uptake of cholera toxin B subunit (CTB), a known substrate for caveolae-mediated endocytosis in other cell types [13,25]. CTB uptake in RCECs was not affected by pretreatment of cells with either filipin or nystatin (Figure 1B), indicating that caveolae may be absent in RCECs.

Immunofluorescence studies: To further validate the possible involvement of clathrin-mediated processes in endocytosis of PLGA nanoparticles, we investigated whether the staining pattern of coumarin-loaded nanoparticle is associated with that of clathrin in RCECs. Figure 2 shows confocal images of RCECs (40x magnification) stained with clathrin HC following 30 min apical uptake of PLGA nanoparticles. As seen in the double-labeling studies (panel D), clathrin staining (red in panel A) is partially overlapped with that of 6-coumarin-loaded nanoparticles (green in panel B), indicating that nanoparticle
uptake may partially associate with clathrin-mediated processes. The fluorescence colocalization pattern for both nanoparticles and clathrin was found both at the plasma membrane and intracellularly, probably due to internalized clathrin-coated vesicles. Immunostaining for caveolae using mouse caveolin-1 monoclonal antibody did not reveal any staining pattern in RCECs (image not shown), fortifying the notion that caveolae-mediated processes are absent in RCECs. These findings were confirmed with magnification of up to 100x, however, for best illustrative quality without compromising image resolution only the 40x magnified specimens are shown.

**Molecular evaluation of clathrin and caveolin gene expression:** The results in Figure 3A illustrate RT-PCR products detected in both RCECs and HEK293 (as control) corresponding to an expected 744 bp product. To verify that this amplified product represented clathrin HC cDNA, the band for clathrin HC cDNA fragment was extracted, sequenced, and found to have 66% and 62% identity to clathrin HC mRNA from human and rat, respectively, using the Clustal W sequence alignment program (Figure 4). To address whether caveolin cDNA was present in RCECs, RT-PCR amplification of the mRNA transcript was performed using cDNA primer sequences designed from conserved caveolin-1 regions. Figure 3B shows that the expected RT-PCR gene product for caveolin-1 (corresponding to 152 bp in size) was not detected in RCECs and rabbit brain tissue, but was detected in several others including the cornea, trachea, cardiac muscles, and HEK cells. The signal for GAPDH was also observed at 488 bp similarly for all tissues tested in Figure 3B for positive control.

To verify the existence of clathrin HC and caveolin-1 at the protein level, western blot analysis of RCEC lysates was performed using mouse monoclonal antibodies. Figure 5A shows that the clathrin HC antibody detected a 180 kDa-sized protein in RCECs and HeLa cells. On the other hand, caveolin-1 antibody could not detect any protein band in RCECs, but did detect a 22 kDa-sized protein in both A431 cells, known to express caveolin-1 abundantly, and rabbit cardiac muscle cells used to demonstrate cross-reactivity of mouse caveolin-1 antibody against rabbit protein (Figure 5B).

**Knockdown of clathrin HC by antisense oligonucleotides:** In order to ascertain the role of clathrin in nanoparticle endocytosis, we devised an antisense oligonucleotide-based strategy to knockdown clathrin HC transcript levels in RCECs and HeLa cells. Figure 6A demonstrates that RCECs transfected with antisense oligonucleotides using Lipofectamine™ reagent shows that the clathrin HC antibody detected a 180 kDa-sized protein in RCECs and HeLa cells. Figure 6A demonstrates that RCECs transfected with antisense oligonucleotides using Lipofectamine™ reagent inhibited clathrin protein expression in a dose dependent manner, with an apparent IC₅₀ of 0.99±0.01 µg/ml. Similar treatment with sense oligonucleotides did not cause any inhibition at the same concentrations tested (not shown). A similar pattern of clathrin protein inhibition occurred in HeLa cells as a result of transfection with antisense oligonucleotides (data not shown). In contrast to earlier pharmacological treatments including the cornea, trachea, cardiac muscles, and HEK cells.

Figure 4. Nucleotide sequence of amplified rabbit clathrin HC gene fragment. The nucleotide sequence of rabbit clathrin HC is compared with known sequences of human and rat. Residues identical to clathrin HC between species are labeled with an asterisk. The nucleotide sequence used for the design of the antisense utilized in clathrin HC knockdown is shown in red (between 290-304 bp of rabbit clathrin HC).
in these transfected RCEC compared to mock transfection (with sense oligonucleotide), which is commensurate with clathrin protein reduction in these cells (Figure 6D).

**DISCUSSION**

Our most significant finding in this study is that rabbit conjunctival epithelial cells express clathrin HC at the gene and protein level, but not caveolin-1. In addition, we have obtained evidence that endocytosis of PLGA nanoparticles in rabbit conjunctival epithelial cells occurs independently of both clathrin HC and caveolin-1, although PLGA nanoparticle uptake may in part take place via clathrin-mediated processes. The existence of an endocytic mechanism for the internalization of PLGA nanoparticles in rabbit conjunctival epithelial cells was previously suggested by the uptake inhibition by cytochalasin D treatment as well as the punctate distribution pattern shown under confocal microscopy (unpublished data). Similar endocytic processes were also described for the uptake of PLGA nanoparticles in other cell types, including vascular smooth muscle cells [26]. However, the mechanism of endocytosis of polymeric nanoparticles into epithelial-type cells specifically has not been elucidated, to our knowledge. Panyam et al. [27] reported that uptake of PLGA nanoparticles in vascular smooth muscle cells was significantly reduced after inhibition of clathrin-mediated pathways, but not for the caveolae-dependent ones. Similar findings were also reported by Huang et al. [28] for the uptake of FITC-chitosan nanoparticles by the A549 cell line. As a result of intracellular K⁺ depletion or treatment of RCECs with hypertonic solution (pharmacological treatments aimed at indirectly inhibiting clathrin-mediated endocytosis) we initially confirmed the involvement of clathrin in nanoparticle uptake. Disruption of clathrin-related endocytic mechanisms in RCEC resulted in 35-45% maximal inhibition of PLGA nanoparticle uptake. In contrast, disruption of caveola-mediated processes with either nystatin or filipin (substrates affecting cholesterol functionality in cells and caveolae structure) did not affect PLGA nanoparticle uptake. Utilizing antisense oligonucleotides as a way to knockdown the protein expression of clathrin HC, our results from endocytosis of PLGA nanoparticle in rabbit conjunctival epithelial cells suggested clathrin HC-independent mechanisms. These findings may indicate that endocytosis of PLGA nanoparticles in rabbit conjunctival epithelial cells occur through multiple mechanisms, out of which clathrin-coated pits may not contribute significantly. It is also possible that PLGA nanoparticles enter epithelial cells through a process similar to adsorptive endocytosis, with nanoparticles being concentrated on membrane pits that are non-coated rather than floating in cell fluid (such as fluid-phase markers). Such complex pathways are poorly understood, but have been attributed in the entry of influenza virus and internalization of interleukin-2 receptors in HeLa cells and lymphocytes, respectively [14,29].

Another possibility may be that the endocytic pathway for nanoparticles bear a resemblance to that of lipid absorption through the intestine or hepatocytes, where particulates enter the cells through the microvilli via endocytosis, transit through the endoplasmic reticulum (ER), and get packaged into vesicles in the Golgi apparatus before fusing with the lateral membrane for exocytosis [30]. Evidence for paracellular and transcellular uptake of nanoparticles into the intestinal epithelium is well documented [31]. While our unpublished data did reveal partial colocalization of staining pattern of PLGA nanoparticle with the Golgi compartment (using Golgin58 as an antibody marker) in RCECs, further studies are required to support Golgi vesicle sorting of nanoparticles into the lateral side of RCECs (unpublished data). It may be pointed out that treatments such as hypertonic and intracellular K⁺ depletion may not be specific toward interrupting formation of clathrin-coated vesicles and may affect other endocytic processes in general. However, one cannot rule out non-specific, random association of nanoparticles in clathrin-coated vesicles.

Clathrin plays an important role in endocytosis of macromolecules and nutrients as well as in membrane trafficking steps, particularly in sorting events in the trans-Golgi network. Although clathrin is thought to be a ubiquitous protein, the existence of clathrin in ocular tissues has only been described in the rat retina and lens epithelial cells [17,18]. Our current study is the first report describing the presence of clathrin mRNA and protein in the rabbit conjunctiva. Earlier studies have highlighted the important role played by clathrin heavy chain, as demonstrated by the decrease in pinocytosis and receptor-mediated endocytosis in clathrin HC deficient CV-1 cells [32]. Previous attempts at disrupting clathrin HC involved targeted gene mutation studies in single cells in eukaryotes or cell lines [33-35]. The use of stably transfected cell lines with either mutant dynamin or antisense overexpression of clathrin HC has been important in estimating the role of clathrin [36,37]. A clathrin knockout model using an antisense approach in primary cultures of epithelial cells is a better approxima-

![Figure 5: Western blot analysis of clathrin HC and caveolin-1 expression.](image-url)

**Figure 5.** Western blot analysis of clathrin HC and caveolin-1 expression. **A:** Expression of clathrin HC in RCECs and HeLa cells (positive control) probed with clathrin HC monoclonal mouse antibody. **B:** Expression of caveolin-1 in A431 cells (positive control) and rabbit cardiac muscle cells, but not in RCECs, was found when probed with caveolin-1 monoclonal mouse antibody. All cell lysates were prepared from confluent cells grown on tissue culture-treated substratum.
tion of physiological situations than the use of cell lines, as we have shown in this study.

Caveolae are particularly abundant in endothelial cells, adipocytes, vascular smooth muscle, fibroblasts, and lung alveolar type I epithelial cells [38]. The functions of caveolae are relatively poorly understood, but they have been implicated in the transcytosis of some molecules (e.g., albumin), cholesterol homeostasis, and signal transduction. For instance, Griffoni et al. [39] have shown that caveolin-1 knockdown impairs angiogenesis in vitro and in vivo. Since caveolin-1 is the most widely investigated of the caveolin proteins (3 isoforms in all) and because over 90% of caveolin-1 is found associated with caveolae, it can be reliably used as a marker for caveolae structures [40]. Our data indicate that rabbit conjunctival epithelial cells do not express caveolin-1 mRNA or protein, as assessed by RT-PCR and western analysis. Our finding for the absence of caveolin-1 in rabbit conjunctival epithelial cells is in contrast to the report by Boulton et al. [41] of positive staining of caveolin-1 in human conjunctival tissue. Structural differences of conjunctival and corneal epithelial tissues between human and rabbit species may explain the apparent species differences [42], and/or the use of whole conjunctival tissue by Boulton and coworkers, which may have resulted in caveolin-1 staining of cells from non-epithelial origin. When we performed western blot analysis on whole conjunctival tissue obtained from pigmented rabbits, the expression of caveolin-1 could be visualized (data not shown). By contrast, carefully isolated conjunctival epithelial cells do not display the expression of caveolin-1 both at the gene and protein levels, unlike the intact tissue. It is worthwhile to point out that gene expression of caveolin-1 in rabbit corneal epithelial tissue is in agreement with a previous report [16] and that the absence of caveolin-1 gene expression in rabbit brain

Figure 6. Knockdown of clathrin HC protein expression. A: Western blot analysis of RCEC lysate following 24 h transfection using Lipofectamine reagent (10 µg/ml) with various concentrations of antisense oligonucleotides targeted against clathrin HC mRNA. Western blot analysis of actin protein expression in the same RCEC lysate was performed as a positive control for antisense oligonucleotide specificity against clathrin HC. Figure 6A represents one typical western blot (n=1). For antisense sequence and source, see methods section. B: Quantitative analysis of clathrin expression normalized against actin protein expression as a function of increasing doses of antisense oligonucleotides in µg. Images and bands were scanned and quantified using Scion Image software and indicated as percent inhibition with respect to clathrin HC levels in untreated samples (mock transfection). Bars represent mean±standard error of the mean (n=4). C: Apical uptake of PLGA nanoparticles (0.5 mg/ml) in RCECs at 37 °C for 30 min following 24 h transfection with antisense oligonucleotides against clathrin HC. Bars represent mean±standard error of the mean (n=4). D: Western blot similar to A above, but with different concentrations of antisense oligonucleotide. E: Basolateral uptake of FITC-transferrin (50 µg/ml) in RCECs at 37 °C for 30 min following 24 h transfection with antisense oligonucleotides against clathrin HC. Bars represent mean±standard error of the mean (n=4). The asterisk denotes significant differences from control (zero antisense oligonucleotide concentration, p<0.01).
tissue (Figure 3B) is consistent with reports of Tang et al. [43], who used rat brain tissue. The expression of caveolin-1 protein in rabbit cardiac muscle cells rules out the possible lack of cross reactivity of mouse caveolin-1 monoclonal antibody with rabbit protein. Our pharmacological data suggest that other caveolins (caveolin-2 or 3) are not likely to be involved in nanoparticle endocytosis in rabbit conjunctival epithelial cells.

In summary, our findings indicate the absence of a clear correlation between endocytosis of PLGA nanoparticles in rabbit conjunctival epithelial cells and the involvement of clathrin HC or caveolin-1 proteins. The absence of a caveolin-1 specific signal in these cells coupled with the lack of changes in PLGA nanoparticle uptake under clathrin HC protein knockdown conditions of RCECs requires further investigation to offer some physiological relevance. It can be suggested that adsorptive endocytosis or macropinocytosis (less likely) may be involved in nanoparticle endocytosis into rabbit conjunctival epithelial cells, which may resemble the pathway of lipid and particulate absorption reported in the intestinal epithelium. As a corollary to our findings for the existence of clathrin in conjunctival epithelial cells, future studies aimed at dissecting the endocytic pathway used by different molecules and the role of clathrin-mediated endocytosis in conjunctival physiology may benefit from our presented data.

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