

Apical-to-Basolateral Transcytosis of Transferrin-Polylysine Conjugates in Caco-2 Cell Monolayers: A Model of Relay Transport of Protein Drug across Epithelial Cells

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Abstract

While the number of therapeutic biologics on the market has dramatically increased in the past decades, oral delivery of protein/peptide drugs is still one of the challenges for biopharmaceutical scientists. Transferrin (Tf), with its receptor-mediated transcytosis mechanism across intestinal epithelium, has been proven as a potential oral delivery carrier for different protein therapeutics. However, one of the major rate-limiting issues of Tf in oral absorption is that Tf Receptor (TfR) is selectively expressed on the basolateral surface instead of the apical surface of the intestinal epithelium, and thus, limits the mucosal transport capacity of Tf across the intestinal epithelium. To overcome this limitation and develop a more efficient oral delivery carrier, a novel transcytosis mechanism was proposed. The proposed transcytosis mechanism is composed of three major steps: apical binding and endocytosis of Tf with a cationic peptide carrier, intracellular cleavage of the linker between peptide and Tf, and basolateral exocytosis by TfR. The feasibility of the proposed approach was demonstrated when Tf was conjugated to poly-L-lysine (PLL) using a pH-sensitive Nitrilo Triacetic Acid (NTA) linker in the presence of nickel (Ni²⁺). The results of Caco-2 transcytosis assay suggested that the transcytosis rate of histidine-tagged Tf could be unidirectionally increased from apical to basolateral compartment when co-incubated with Ni²⁺-NTA-PLL.

Keywords

Transferrin; Proteinoral Delivery; Transcytosis; Poly-L-Lysine; Nitrilotriacetic Acid Linker.

Introduction

Protein and peptide biologics have become an important category of drugs for the treatment of human diseases [1]. One of the limitations in developing proteins and peptides into therapeutic drugs is the lack of a general method for their non-invasive delivery [2,3]. Among all non-invasive routes, the oral delivery of proteins and peptides has long been recognized as an ultimate challenge in drug delivery [4]. The advantages in oral delivery of protein drugs have been well-recognized, and the feasibility of oral delivery of proteins has been demonstrated in various technologies [5]. However, there is no FDA-approved drug delivery system currently available on the market that can enhance the oral bioavailability of protein drug [6]. One of the hurdles in developing oral protein delivery system is that many of the delivery techniques are based on non-physiological approaches, such as using penetration enhancers and surfactants, that may cause irritation or side-effects in the gastrointestinal (GI) tract, especially in chronic applications [7]. Therefore, it is desirable that the GI absorption of protein drugs relies on natural mechanisms in GI physiology for transcellular protein transport. Transcytosis is the major physiological process for the release of proteins across either epithelial or endothelial cell layers [8]. Conceivably, transcytosis of a protein drug across intestinal epithelial cells from the mucosal to serosal surface via a physiological pathway is an ideal route for oral protein drug delivery system.

Several studies in the past decades have been focused on developing transcytosis-based systems for oral protein drug delivery. In general, there are two different approaches in those studies, i.e., 1) Receptor-mediated transcytosis; such as transferrin(Tf), vitamin B-12, and cholera toxin B subunits [9-11] and 2) Adsorptive transcytosis such as protamine and cell-penetrating peptides [12]. Even though limited success in oral bioavailability of protein drugs has been achieved in those reports, a highly efficient transcytotic pathway is yet to be identified. Under physiological conditions, the release of proteins from epithelial cell layers, such as the secretion of IgA via pIgR-mediated transcytosis, is mostly from serosal-to-mucosal surfaces [13]. In addition, the polarity of epithelial cells restricts the receptor distribution between serosal and mucosal membranes [14] and renders receptor-mediated transcytosis in the mucosal-to-serosal direction inefficient. On the other hand, adsorptive transcytosis relies on non-specific binding of the protein molecule on the surface of the

Article Information

DOI: 10.31021/ijbs.20181107
Article Type: Research Article
Journal Type: Open Access
Volume: 1 Issue: 1
Manuscript ID: IJBS-1-107
Publisher: Boffin Access Limited.

Received Date: October 26, 2017
Accepted Date: November 28, 2017
Published Date: January 31, 2018

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Citation: Chen YS, Zaro JL, Shen WC. Apical-to-Basolateral Transcytosis of Transferrin-Polylysine Conjugates in Caco-2 Cell Monolayers: A Model of Relay Transport of Protein Drug across Epithelial Cells. Int J Biopharm Sci. 2018 Jan;1(1):107

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epithelial mucosal membrane. Even with a very high rate of surface binding and endocytosis, most of the non-specific membrane-bound protein will be sorted to the lysosomes and degraded, rather than being transcytosed to the serosal membrane [15]. Therefore, the low efficiency in mucosal-to-serosal transcytosis in intestinal epithelial cells is a major hurdle in the development of transcytosis-based oral protein delivery.

In this report, we propose a novel approach to increase the transcytosis of a protein molecule across the epithelial cells. The approach is based on the increase of mucosal uptake of the protein molecules via adsorptive endocytosis and the serosal release of the protein molecules via receptor-mediated recycling. This transport process involves an intracellular exchange of the protein drug from an adsorptive carrier, e. g., cationic polylysine, to a receptor-mediated carrier, e.g., Tf inside the common endosomes [16]. Such a "relay transcytosis" mechanism has been found in the transcytotic process of several macromolecular ligands in GI epithelium. For example, cobalamin (vitamin B-12) has been shown to be internalized as the intrinsic factor complex via the intrinsic factor receptor from the mucosal membranes of intestinal epithelial cells. Once internalized, the complex is degraded and cobalamin is then released from lysosomes to cytoplasm as the transcobalamin complex. The transcobalamin complex is then released to the blood circulation from the serosal membrane [9]. To demonstrate the proposed mechanism, Tf and poly-L-lysine (PLL) conjugated by two different intracellular cleavable linkers, a disulfide and an acid liable linker, were investigated for the binding and transcytosis in cultured Caco-2 cell monolayers.

Methods

Cell culture

All the binding and transcytosis experiments were performed on Caco-2 cells, obtained from the American Type Culture Collection (ATCC). Caco-2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 20% (v/v) Fetal Bovine Serum (FBS), 2 mM L-glutamine, 50 unit/ml penicillin, 50 µg/ml streptomycin and 0.1 mM non-essential amino acids. HEK293 cells purchased from ATCC were used for the production of recombinant protein, and were grown in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 50 unit/ml penicillin and 50 µg/ml streptomycin.

For transcytosis assays, Caco-2 cells were seeded on polycarbonate membrane filters (0.4 µm pore size) in 6-well Transwell plates (Corning, Corning, New York) as previously described [13]. The Caco-2 cells were grown for 21 days after confluence, with culture medium replaced every 2 days as well as 24 h before each experiment. The Transepithelial Electrical Resistance (TEER) levels were measured using an epithelial volt ohmmeter (World Precision Instruments, West Haven, CT) as an indicator of tight junction integrity and the differentiation of Caco-2 cells into mature enterocyte-like cells. The Transwell membranes with TEER levels of 500 ohms·cm² or higher were used for transcytosis assays.

Preparation of disulfide-linked Tf-PLL Conjugates (Tf-ss-PLL)

Human serum Tf (Sigma) was covalently linked to PLL (n=21 lysine residues, synthesized by Alamanda Polymers, Huntsville, AL) via a reducible disulfide linkage with a bifunctional cross-linking reagent, N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP, Thermo Fisher Scientific, Canoga Park, CA). First, Tf and PLL were each reacted with SPDP (molar ratio = 1:4 and 1:2, respectively) at 4 °C for 4 h followed by purification using Sephadex G50 and G25, respectively, size exclusion chromatography (GE Healthcare, Piscataway, NJ) with PBS as the mobile phase Figure 1. The modification ratio of the resultant products, Tf-PDP and PLL-PDP was determined to be 1:3 Tf: PDP and 1:1.5 PLL:PDP. Tf-PDP was then treated with dithiothreitol (DTT) to expose sulfhydryl groups, and purified using size exclusion chromatography. Sulfhydryl-containing Tf was reacted with PLL-PDP at 4 °C for 24 h to generate disulfide bond-linked Tf-PLL conjugates

(Tf-ss-PLL). The 2,4,6-Tri Nitro Benzene Sulfonic Acid (TNBSA, Sigma) assay using a standard curve generated with N α -Acetyl-L-lysine methyl ester hydrochloride was applied following the manufacturer's instructions to estimate the PLL modification of Tf in Tf-ss-PLL. Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining were applied to further analyze the Tf-ss-PLL product Figure 3.

Preparation of Tf-PLL conjugates with an acid liable linker (Tf-NTA-PLL)

The pH-sensitive nitrilotriacetic (NTA) linker was applied for Tf-PLL conjugation. It has been demonstrated that the coordination bonds between the polyhistidine-tag and NTA-coordinated nickel ion dissociate at pH 6 or lower [33]. Human Tf with a hexahistidine-tag on the N-terminus (Tf-6xHis) was recombinantly expressed and purified as previously described [17]. Briefly, HEK 293 cells (ATCC, Manassas, VA) were transiently transfected with pc DNA 3.1(+) DNA plasmid containing the gene of Tf-6xHis via polyethylenimine-mediated transfection. The conditioned CD293 medium (Thermo Fisher Scientific Inc.) was collected twice (Day 4 and Day 7) post-transfection, and concentrated using tangential flow filtration (Millipore). The column packed with nickel nitrilotriacetic acid agarose (Thermo Fisher Scientific Inc.) was used for protein purification.

NTA-modified PLL (NTA-PLL) was synthesized as shown in Figure. 2. First, N α , N α -Bis(carboxymethyl)-L-lysine (Sigma) was reacted with succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB, Thermo Fisher Scientific Inc.) at a molar ratio of 6:1 at room temperature for 4 h to generate NTA-MPB. PLL was reacted with SPDP at a molar ratio of 1:2 at 4°C for 4 h, and subsequently purified by G25 size exclusion chromatography. The final ratio of PLL:PDP was 1:1.8. PLL-PDP was reduced by the treatment with 25 mM DTT to release the thiopyridine group and generate the free sulfhydryl group. The sulfhydryl-containing PLL was then reacted with NTA-MPB to form NTA-PLL. The excess NTA-MPB was removed using G25 size exclusion chromatography. The Tf-NTA-PLL complex was then prepared by mixing Tf-6xHis with NTA-PLL and NiSO₄ (Sigma).

Radioactive iodination of protein samples

The purified protein samples, including Tf, Tf-ss-PLL and Tf-6xHis, were labeled with radioactive ¹²⁵I using Chloramine T method as previously described [18]. In brief, 1 mg of protein was mixed and reacted with Na¹²⁵I (PerkinElmer, Waltham, MA) and oxidizing agent Chloramine-T (Sigma) on ice for 5 min. Sodium metabisulfite was then added and the reaction was incubated on ice for another 5 min. Excess potassium iodide was added to stop the radio labeling reaction. Finally, the ¹²⁵I -labeled protein was purified by G-50 size exclusion chromatography using PBS as the mobile phase, and the radioactivity of ¹²⁵I-labeled protein in each fraction was counted using a gamma counter (Packard, Downers Grove, IL).

Caco-2 binding assays

Binding assays on Caco-2 cells were performed to determine the increased binding of Tf to cell membrane due to the conjugation of PLL. Caco-2 cells were seeded in 6-well plates; the experiments were performed after 3 days of seeding when cell confluence was reached. Caco-2 cells in 6-well plates were first incubated in Hank's Balanced Salt Solution (HBSS) at 37 °C for 30 min to remove the serum endogenous Tf. After the incubation, cell monolayers were washed twice with 1 mL of ice-cold HBSS, and treated with the dosing solutions containing ¹²⁵I -labeled proteins in HBSS at 2 µg/mL. After 2 h of incubation at 4 °C, the dosing solutions were removed, and the cell monolayers were washed three times with ice-cold PBS. The cells were then dissolved in 1 mL of 1 N NaOH, and radioactivity of the cell lysates was counted using a gamma counter. The Micro BCA Protein Assay Kit (Thermo Fisher Scientific Inc.) was used to measure the concentration of cell lysates for normalizing the results.

pH-sensitive dissociation between Tf-6xHis and Ni²⁺-NTA-PLL

¹²⁵I-labelled Tf-6xHis was first co incubated with NTA-PLL plus nickel ions (Tf-6xHis:NTA-PLL:Ni²⁺ ratio = 1:10:40) in PBS

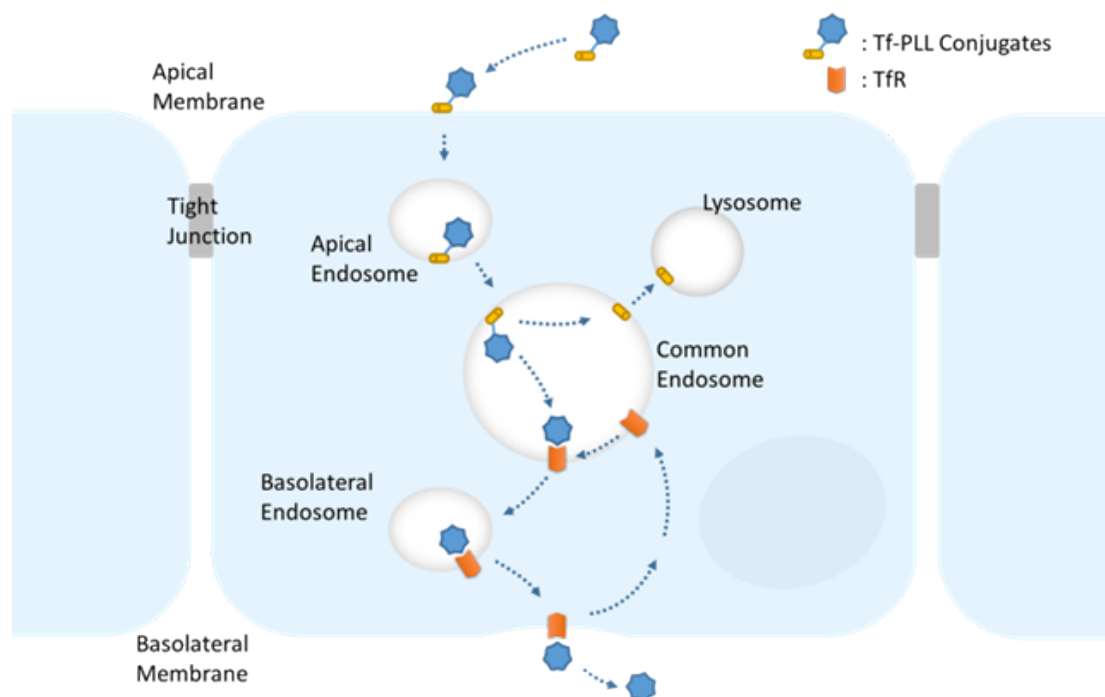


Figure 1: Transcytosis of Tf-PLL conjugates across Caco-2 epithelial cells. Tf-PLL conjugates with an endosomal cleavable linker can be bound to the apical membrane of epithelial cells and internalized into cells via a non-specific adsorptive endocytosis mechanism provided by the positive charges of PLL. The endocytosed Tf-PLL conjugate can be distributed to common endosome that is also accessible to basolaterally endocytosed and recycled Tf and TFR. In the common endosome, Tf will be dissociated from PLL by the cleavage of linker, bind the unoccupied TFR, and subsequently be released to basolateral surface of epithelium by TFR-mediated recycling pathway. On the other hand, the membrane-bound PLL in the endosomes will be eventually transported to and degraded in the lysosomes.

(pH 7.4) at 25 °C for 30 min, and then loaded into cation exchange microcentrifuge spin columns (Thermo Fisher Scientific Inc.). After spinning down the mixture and removing the flow-through, the columns were washed twice with 200 μ L of PBS, and subsequently eluted with 200 μ L of citric acid- Na_2HPO_4 buffers at various pH from 7.0 to 5.5. The radioactivity of each eluate was counted using a gamma counter.

Caco-2 transcytosis assay

Prior to the transcytosis experiments, cell monolayers were washed and then incubated with HBSS containing 10 mM HEPES (pH 7.4) at 37°C for 30 min to remove the serum Tf. Then, the dosing solution (2 μ g/mL ^{125}I -labeled proteins in HBSS containing 10 mM HEPES, pH 7.4) was added to the apical or basolateral compartment of filter-growth Caco-2 cells. After 8-h incubation at 37°C, the buffers from the opposite compartment containing the transcytosed ^{125}I -labeled proteins were collected; the radioactivity levels were counted as total transcytosed proteins that included both intact and degraded proteins. The collected samples were subsequently subjected to 15% trichloroacetic acid (TCA) precipitation [19]. The radioactivity in the protein precipitate was measured as intact transcytosed protein. The TEER of cell monolayers was measured using an epithelial volt ohmmeter before and after the treatments to ensure the integrity of tight junctions. The Caco-2 cell monolayers on membrane supports were washed three times with ice-cold HBSS and cut off from the Transwell inserts. The radioactivity of the collected membrane was counted to estimate the amount of cell-associated protein.

Results

Preparation of Tf-ss-PLL and Tf-NTA-PLL

In order to evaluate the disulfide linkage in Tf-ss-PLL, the number of primary amine groups was measured using TNBSA assay. The results in Figure 3a showed that Tf-ss-PLL group contained an

additional 60 amine groups per molecule when compared with Tf-PDP. The increased number of amine groups indicated that Tf was successfully conjugated with PLL, and the conjugation ratio of Tf:PLL was about 1:3 which was consistent with the ratio of Tf:PDP (1:3). SDS-PAGE followed by Coomassie blue staining was used to further confirm the disulfide bond conjugation. From the 10% SDS-PAGE as shown in Figure 3b, a slight increase of molecular weight can be noticed on the major band of non-reduced Tf-ss-PLL when compared with non-reduced Tf. Some Tf oligomers, possibly due to the multiple PDP modifications of PLL, were also noticed. However, after the treatment with DTT, there is no difference between Tf and Tf-ss-PLL groups. For Tf-NTA-PLL conjugate, the conjugation between Tf-6xHis and Ni^{2+} -NTA-PLL was confirmed using Caco-2 cell binding assay.

Caco-2 binding assay of Tf-ss-PLL

Caco-2 monolayers were treated with dosing solutions containing ^{125}I -labeled Tf or Tf-ss-PLL at 4°C to compare the cell membrane binding between these two proteins (Figure 4). Based on the results, it was confirmed that the cell membrane binding of Tf could be significantly enhanced after covalent conjugation to PLL. After 2-h incubation, the cell membrane-bound amount of Tf-ss-PLL (9192.17 ± 658.05 ng/mg total cell protein) was 18.3 fold higher than that of Tf (500.84 ± 92.84 ng/mg total cell protein). In addition, for ^{125}I -Tf-ss-PLL treated groups with the increased concentration of cysteamine, the radioactivity levels in cell lysates were gradually decreased (Figure 4). Since only the tyrosine residues of Tf were labeled with radioactive ^{125}I , the results further confirmed that the ^{125}I -Tf can be released from PLL when ^{125}I -Tf-ss-PLL was exposed to a reductive environment.

Caco-2 binding and pH-sensitive dissociation of Tf-NTA-PLL

Caco-2 binding assay was performed to confirm the successful conjugation between Tf-6xHis and Ni^{2+} -NTA-PLL via coordination

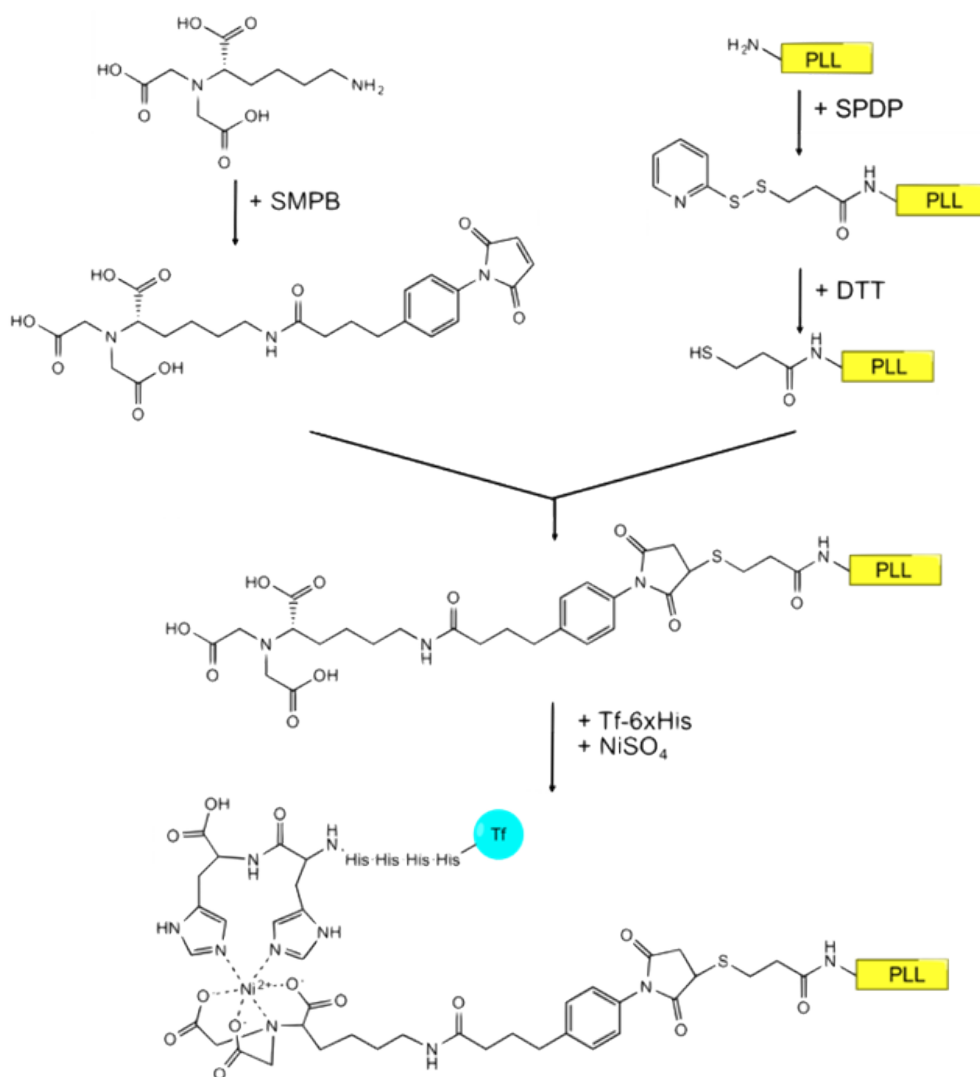


Figure 2: Preparation of Tf-NTA-PLL conjugates. α , α - Bis (carboxymethyl)-L-lysine was reacted with SMPB to produce NTA-MPB. PLL was reacted with SPDP to produce PLL-PDP, followed by treated with DTT to generate free Sulphydryl group. Sulphydryl-containing PLL was subsequently reacted with NTA-MPB to form NTA-PLL. Coordination bonds between Tf-6xHis and NTA-PLL were formed by directly mixing NTA-PLL with Tf-6xHis and NiSO_4 .

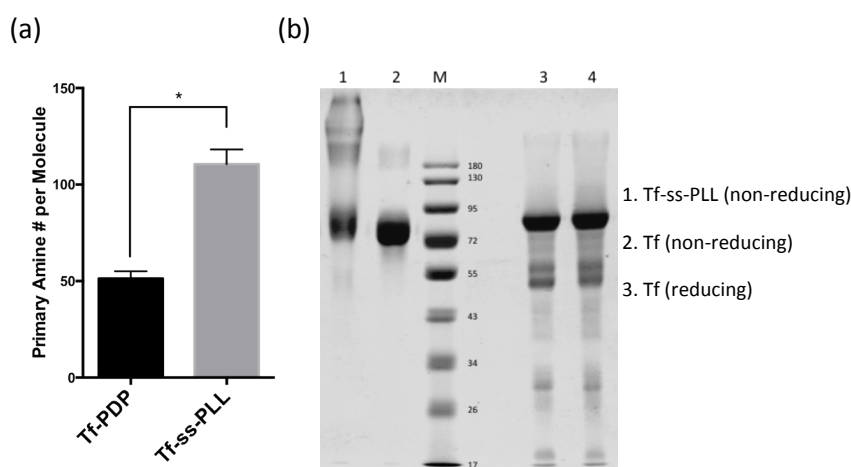


Figure 3: Characterization of Tf-ss-PLL. (a) The number of primary amino groups per molecule was determined by TNBSA assay. 100 $\mu\text{g}/\text{mL}$ Tf-PDP or Tf-ss-PLL was reacted with TNBSA followed by 2-h incubation at 37 $^{\circ}\text{C}$. 10% SDS and 1N HCl were added into the samples. The number of amines in the sample was determined by measuring the absorbance at 335 nm and using a standard curve of α -Acetyl-L-lysine methyl ester hydrochloride. Single asterisks (*) indicates significance with $P < 0.05$, as determined by the Student's t-test ($n=3$) (b) 10 % SDS-PAGE followed by Coomassie blue staining. 6 μg of indicated proteins with or without the reducing agent DTT were loaded.

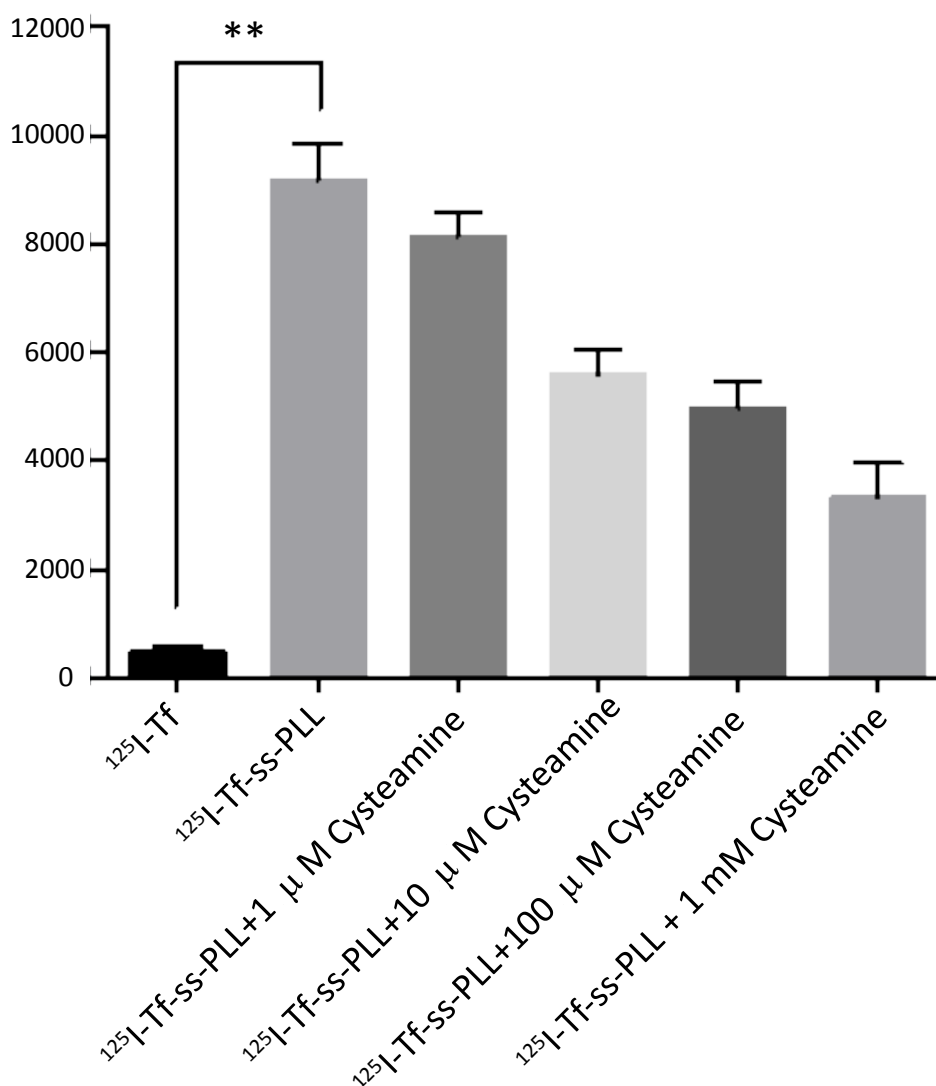


Figure 4: Caco-2 binding assay of Tf-ss-PLL. Caco-2 cells were treated with HBSS containing 10 $\mu\text{g/mL}$ $^{125}\text{I-Tf}$ or $^{125}\text{I-Tf-ss-PLL}$ at 4 °C for 2 h. Serial dilutions of cysteamine from 1 μM to 1 mM were added to the cell culture medium. The data represent counted radioactivity of cell lysates normalized by total cell protein amount determined using MicroBCA assay. Double asterisks (**) indicates significance with $P < 0.01$ as determined by the Student's t-test ($n=3$)

bonds (Figure 5a). Based on the results, the control group without NTA modification on PLL (187.71 ± 19.96 ng/mg cell protein) or without nickel ions (199.03 ± 16.68 ng/mg cell protein) displayed no significant increase on cellular binding of $^{125}\text{I-Tf-6xHis}$ when compared to $^{125}\text{I-Tf-6xHis}$ alone (165.28 ± 25.60 ng/mg cell protein). On the other hand, the binding of $^{125}\text{I-Tf-6xHis}$ on the cell membrane was significantly increased when coincubated with NTA-PLL plus nickel ions (Tf-6xHis:NTA-PLL: Ni^{2+} ratio = 1:10:40; 407.08 ± 6.95 ng/mg cell protein). In addition, the binding was further increased in the presence of higher concentration of NTA-PLL and Ni^{2+} (Tf-6xHis:NTA-PLL: Ni^{2+} ratio = 1:40:160; 549.64 ± 32.91 ng/mg cell protein).

To demonstrate the pH-sensitive dissociation between Tf-6xHis and Ni^{2+} -NTA-PLL, the same amount of Tf-NTA-PLL was first immobilized on the cation ion exchange spin columns and eluted with buffers at different pH, respectively (Figure. 5b). The significant increase of the radioactivity in the pH 5.5 eluate, as compared with at pH 6.0 or higher, indicated that the pH sensitive dissociation occurred between pH 5.5 and 6.0.

Transcytosis Assay of Tf-ss-PLL

In this study, the effect of disulfide bond conjugated-PLL on the apical-to-basolateral (A-to-B) transcytosis of Tf was investigated in

23-day cultured Caco-2 cells in Transwell plates (Figure. 6). After the 8-h treatment, the cell monolayer apically treated with $^{125}\text{I-Tf-ss-PLL}$ (73.32 ± 17.32 ng) exhibited 8.74-fold higher cell-associated protein, compared with the $^{125}\text{I-Tf}$ group (8.43 ± 1.23 ng). Also, $^{125}\text{I-Tf-ss-PLL}$ (37.32 ± 1.75 ng) displayed a 2.93-fold higher amount of degraded A-to-B transcytosed protein than $^{125}\text{I-Tf}$ (12.71 ± 0.80 ng). However, after the TCA precipitation, there is no significant difference between the amounts of transcytosed intact protein of these two groups ($p = 0.053$, $n=3$). The amount of transcytosed intact protein on $^{125}\text{I-Tf-ss-PLL}$ group (1.81 ± 0.41 ng) was even lower than that of $^{125}\text{I-Tf}$ group (2.47 ± 0.03 ng). No significant decrease was noticed on the TEER of each membrane after the 8-h treatment.

Transcytosis Assay of Tf-NTA-PLL

Transcytosis assays were conducted to investigate the transcytosis rate of Tf-NTA-PLL (Figure. 7). After the 8-h treatment, the Caco-2 cell monolayer treated with 2 $\mu\text{g/mL}$ $^{125}\text{I-Tf-His}$ showed significantly higher amount of cell-associated protein when coincubated with Ni^{2+} -NTA-PLL (A-to-B: 5.68-fold higher; B-to-A: 5.45-fold higher). However, unlike Tf-ss-PLL, the increase of $^{125}\text{I-Tf-6xHis}$ uptake did not cause the increase of protein degradation. Also, the radioactivity from precipitated pellets of TCA-treated apical or Basolateral media

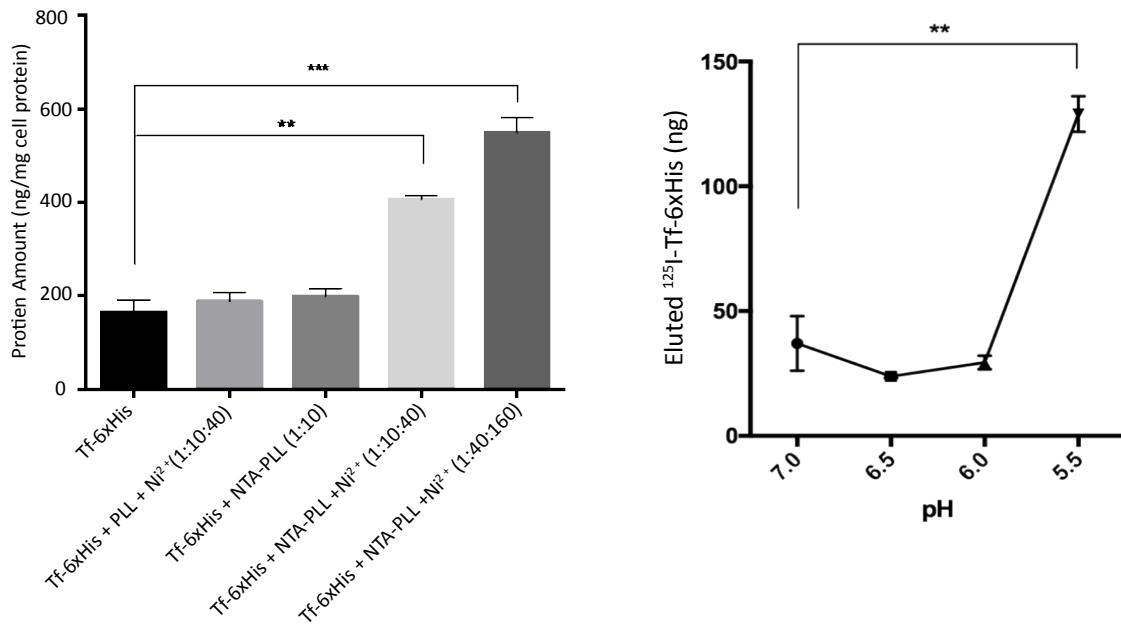


Figure 5: Caco-2 binding assay and pH sensitivity test of Tf-NTA-PLL (a) Caco-2 cells in 6-well plated were incubated with HBSS containing 0.8 µg/mL of ¹²⁵I-Tf-6xHis alone, or in the absence or presence of PLL, NTA-PLL or NiSO₄ at 4 °C for 2 h. The data represent the radioactivity levels of cell lysates normalized by total cell protein amount determined using Micro BCA assay. (b) Cation exchange spin columns loaded with the same amount of ¹²⁵I-Tf-NTA-PLL were eluted with 200 µL of buffers at pH 7.0 to 5.5. The data represent the amount of ¹²⁵I-Tf-6xHis in each eluted sample. The double asterisks (**) and triple asterisks (***) indicated P < 0.01 and 0.001, respectively, as determined by the Student’s t-test (n=3)

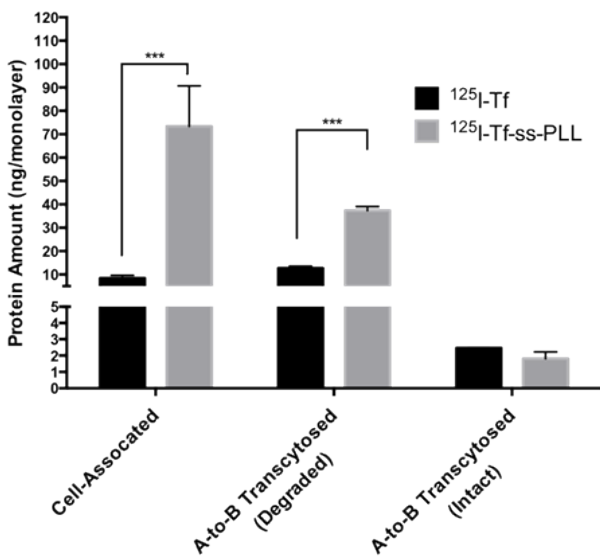


Figure 6: Transcytosis assay of Tf-ss-PLL. Caco-2 cell monolayers grown on 6-well Transwell inserts were apically treated with 2 µg/mL ¹²⁵I-Tf or ¹²⁵I-Tf-ss-PLL. After 8-h incubation at 37 °C, samples were collected from the basolateral compartment, and subjected 15% TCA precipitation. The radioactivity of cell monolayer on the membrane support, collected basolateral solution and TCA-precipitated pellet were counted by a gamma counter as the amount of cell-associated protein, degraded transcytosed protein and intact transcytosed protein, respectively. Triple asterisks (***) indicated P < 0.01 and 0.001, respectively, as determined by the Student’s t-test analysis (n=3).

indicated that ¹²⁵I-Tf-6xHis with NTA-PLL and nickel ions had a 2.68-fold higher amount of intact protein transcytosed in the A-to-B direction compared to ¹²⁵I-Tf-6xHis alone. No statistically significant increase has been noticed for the amount of B-to-A transcytosis of ¹²⁵I-Tf-6xHis-Ni²⁺-NTA-PLL compared to ¹²⁵I-Tf-6xHis. No decrease of TEER was detected after the 8-h for any of the treatments.

Discussion

In this present report, we have conjugated polycationic PLL to Tf with two different cleavable linkers to investigate whether the transcytosis of Tf could be enhanced. This proposed transcytosis mechanism, as shown on Figure 1, was inspired by some previous reports. First, in several natural cases, transcytosis of molecule across epithelial/endothelial barrier can be achieved by incorporating two separated endocytic and exocytic mechanisms. For example, in human antenatal period, maternal IgG is transported across the syncytiotrophoblast by fluid phase endocytosis from maternal blood, followed by neonatal Fc receptor-mediated exocytosis to fetal circulation [20]. Second, in epithelial cells, apical-internalized endosomes could reach the compartment, i.e. common endosomes or sub apical endosomes that is also accessible for basolateral-internalized endosomes [21]. More concrete evidence from Hughson’s previous results has shown that, on polarized Caco-2 cells, apically endocytosed horseradish peroxidase was able to be transported to the same intracellular compartment that contained basolaterally endocytosed anti-Tf receptor (TfR) antibody [22]. Therefore, this finding above supports the feasibility to utilize these common endosomes as transfer stations for Tf-based drug transcytosis.

In this study, PLL was chosen to increase the mucosal uptake of Tf due to several reasons. First, PLL has multiple amine groups on the side chain of lysine residues which allow for convenient modification by commercial crosslinkers, such as SPDP and SMPB. Second, unlike other arginine-rich CPPs, PLL has been shown to be internalized predominantly by endocytosis mechanism rather than membrane transduction[23]. Also, it has shown that PLL exhibited similar endocytosis mechanism as horseradish peroxidase but with

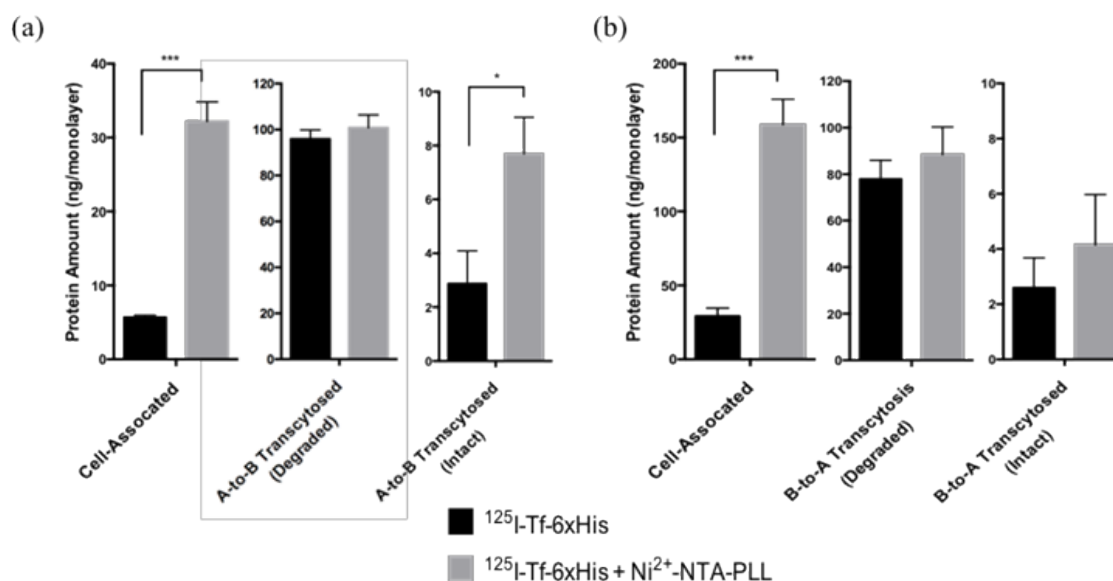


Figure 7: Transcytosis assays of Tf-NTA-PLL. Caco-2 cell monolayers grown on 6-well Transwell were apically (a) or basolaterally (b) treated with $2 \mu\text{g/mL}$ ^{125}I -Tf-6xHis in the presence or absence of NTA-PLL and NiSO_4 (molecular ratio of Tf-6xHis:NTA-PLL: Ni^{2+} = 1:50:200). After 8-h incubation at 37°C , the medium collected from the opposite compartment was subjected 15% TCA precipitation. The radioactivity of cell monolayer on the membrane supports and TCA-precipitated pellets was counted by a gamma counter to estimate the amount of cell-associated protein and intact transcytosed protein, respectively. No change of TEER was observed after the 8-h treatments. Single asterisks (*) and triple asterisks (***) indicated $P < 0.05$ and 0.001 , respectively, as determined by the Student's t-test analysis ($n=3$).

much higher endocytosis rate [24]. Therefore, when conjugated to PLL, Tf-ss-PLL could be apically internalized into epithelium via PLL-mediated absorptive endocytosis process, and subsequently transported to the common endosomes where the basolaterally endocytosed and recycled TfR exists.

Based on our results, the selective dissociation of Tf and PLL in the common endosome are a critical step which allows internalized Tf to reach the TfR in basolateral recycling pathway. In this report, two commonly used cleavable linkers were applied [25]. These two different linkers brought totally different results on the transcytosis studies. The cleavage of disulfide bond linkers depends on the reductive environment during the endocytic pathway [26]. Tf-ss-PLL displayed a much stronger cellular binding and uptake, and an increased amount of A-to-B transcytosis of ^{125}I -labeled material compared to free Tf. However, the A-to-B transcytosis of intact Tf in Tf-ss-PLL was not significantly different than Tf (Figure. 6). These results suggested that most of the internalized Tf-ss-PLL was degraded by the lysosomal enzymes and then released to the cell culture medium. Therefore, it is likely that the disulfide bond linker could not be cleaved efficiently in the endosomes following internalization [27], and that Tf-ss-PLL followed the degradative pathway of PLL to lysosomes, rather than being routed through the non-degradative Tf-pathway of recycling and/or transcytosis.

As another approach, an acid liable linker between Tf and PLL, designed to be released in the mildly acidic pH of endosomal vesicles, was also evaluated. Currently, the most commonly used acid liable linker for drug-carrier conjugation is the hydrazone linker [28]. In this project, a hydrazone linker was also tested to conjugate Tf and PLL. However, the result from the transcytosis assay using hydrazone-conjugated Tf-PLL was similar to that of Tf-ss-PLL. The low transcytosis of hydrazone-conjugated Tf-PLL might be also due to the inefficient cleavage under endosomal pH due to the slow hydrolysis. This assumption is supported by previous studies using a hydrazone linker, where it was shown that only 50% cleavage after 6-h incubation at $37^\circ\text{C}/\text{pH } 5$ was observed [29]. Therefore, to overcome the issue, another acid liable linker, Ni^{2+} -NTA linker, was selected in this report.

His-tagged technology is one of the major approaches for protein purification in biotechnology [30]. The potential application of using the coordinating interaction between polyhistidine-tag and Ni^{2+} -NTA group as a pH sensitive linker for drug delivery has been exploited recently [33]. Unlike the hydrazone linker which is cleaved via a chemical hydrolysis process, the dissociation between His-tag and Ni^{2+} -NTA group relies on the protonation of histidine (pKa 6.0) (Figure. 5b). Thus, this NTA linkage can be rapidly dissociated in the common endosomes (pH 5.8) [31,32]. Such a pH-dependent dissociation can efficiently release Tf from PLL to avoid the lysosomal degradation.

The results demonstrated that co incubation of His-Tagged Tf (Tf-6His) with Ni^{2+} -NTA-PLL could significantly increase its binding and uptake when dosed on either the apical or basolateral side of the epithelial cell surface. However, only A-to-B transcytosis of intact protein was statistically increased (Figure 7). The low B-to-A transcytosis was most likely due to the nature of the Tf-TfR transcytosis/recycling pathway [33]. While it has been shown that TfRs exist in higher amounts on the basolateral membrane, they are majorly recycled back to the basolateral surface rather than transcytosed to the apical membrane [13]. Therefore, the results suggested that the strategy could be only applied to improve the transcytosis of cargos from apical to basolateral side. Also, the result indirectly implies that the TfR-mediated recycling was a limiting step in the proposed transcytosis process.

Results from this report support the hypothesis that a protein, and possibly other macromolecules, can be transported across cellular barriers via a reply transcytosis pathway. This transcellular transport mechanism can be further investigated for noninvasive delivery of macromolecular drugs where the cellular barrier, such as intestinal epithelium and blood-brain barrier, is a limiting factor for the bioavailability [34]. Our results indicate that a fast dissociation between Tf and PLL after the internalization from the apical membrane is an essential step to allow Tf, as a drug or drug carrier, to bind to the TfR in the intracellular compartments and to be transcytosed to the basolateral surface via TfR-recycling pathway. However, the highly pH-sensitive Ni :NTA binding between Tf and PLL

as described in this report cannot be applied to the *in vivo* studies due to the toxicity and carcinogenicity of nickel ion. Therefore, other appropriate linkers, which can be cleaved efficiently and rapidly under the intra-endosomal conditions, should be explored for the design of a practical relay transcytosis pathway for the non-invasive protein drug delivery.

Conclusion

The potential use of Tf and PLL for transcytosis across epithelial monolayers is restricted by the low apical expression level of TfR and low basolateral escape of adsorptive endocytic vesicles, respectively. In this report, Tf and PLL were conjugated together to evaluate a novel "relay transcytosis" mechanism, combining the high apical adsorptive endocytosis of PLL and the capability of efficient basolateral release of Tf via the TfR-recycling/transcytosis pathway. The results from this report demonstrated that the A-to-B intestinal epithelial transcytosis of Tf, when conjugated to PLL with a pH sensitive NTA linker, could be significantly increased. Therefore, this carrier system has the potential to be utilized in enabling the oral delivery of biomolecules.

References

1. Leader B, Baca QJ and Golan DE. Protein therapeutics: a summary and pharmacological classification. *Nat Rev Drug Discov.* 2008 Jan;7(1):21-39.
2. Moeller EH, Jorgensen L. Alternative routes of administration for systemic delivery of protein pharmaceuticals. *Drug Discovery Today: Technologies.* 2008;5(2-3):89-94.
3. Melanie B. Transferrin' the load. *Nature reviews. Drug discovery.* 2005 July;4 (7):537
4. Goldberg M, Gomez-Orellana I. Challenges for the oral delivery of macromolecules. *Nat Rev Drug Discov.* 2003 Apr;2(4):289-295.
5. Park K, Kwon IC, Park K. Oral protein delivery: Current status and future prospect. *Reactive and Functional Polymers.* 2011;71(3):280-287.
6. Shen WC. Oral peptide and protein delivery: unfulfilled promises? *Drug Discovery Today* 8. 2003;(14): 607-608.
7. Kompella UB, Lee VH. Delivery systems for penetration enhancement of peptide and protein drugs: design considerations. *Advanced Drug Delivery Reviews.* 2001;46(1-3):211-245.
8. Tuma PL, Hubbard AL. Transcytosis: crossing cellular barriers. *Physiological Reviews.* 2003;83 (3):871-932.
9. Shah D, Shen WC. Transcellular delivery of an insulin-transferrin conjugates in enterocyte-like Caco-2 cells. *J Pharm Sci.* 1996 Dec;85(12):1306-1311.
10. Ramanujam KS, Seetharam S, Ramasamy M, Seetharam B. Expression of cobalamin transport proteins and cobalamin transcytosis by colon adenocarcinoma cells. *Am J Physiol.* 1991 Mar;260(3 Pt 1):G416-22.
11. Lencer WI, Moe S, Rufo PA, Madara JL. Transcytosis of cholera toxin subunits across model human intestinal epithelia. *Proc Natl Acad Sci U S A.* 1995 Oct;92(22):10094-10098.
12. He H, Ye J, Sheng J, Wang J, Huang Y, et al. Overcoming oral insulin delivery barriers: application of cell penetrating peptide and silica-based nanoporous composites. *Frontiers of Chemical Science and Engineering.* 2013 Mar;7(1):9-19.
13. Su T, Bryant DM, Luton F, Verges M, Ulrich SM, et al. A kinase cascade leading to Rab11-FIP5 controls transcytosis of the polymeric immunoglobulin receptor. *Nat Cell Biol.* 2010 Dec;12(12):1143-1153. doi: 10.1038/ncb2118.
14. Shah D, Shen WC. The Establishment of Polarity and Enhanced Transcytosis of Transferrin Receptors in Enterocyte-like Caco-2 Cells. *J Drug Target.* 1994;2(2):93-99.
15. Tréhin R, Nielsen, HM, Jahnke HG, Krauss U, Beck-Sickingler AG, et al. Metabolic cleavage of cell-penetrating peptides in contact with epithelial models: human calcitonin (hCT)-derived peptides, Tat(47-57) and penetratin(43-58). *Biochem J.* 2004 Sep;382(Pt 3):945-956.
16. Hoekstra D. The subapical compartment: a traffic center in membrane polarity development. *J Cell Sci.* 2004 May 1;117(Pt 11):2183-2192.
17. Sonoda S, Schlamowitz M. Studies of 125I trace labeling of immunoglobulin G by chloramine-T. *Immunochemistry* 1970 Nov;7(11): 885-898.
18. Rajalingam D, Loftis C, Xu JJ, Kumar TKS. Trichloroacetic acid-induced protein precipitation involves the reversible association of a stable partially structured intermediate. *Protein science: a publication of the Protein Society.* 2009 May;18(5):980-993. doi: 10.1002/pro.108.
19. Roopenian DC and Akilesh S. FcRn: the neonatal Fc receptor comes of age. *Nature Reviews Immunology.* 2007;7(9):715-725.
20. Hoekstra D, 2004b. The subapical compartment: a traffic center in membrane polarity development. *J Cell Sci.* 2004 May 1;117(Pt 11):2183-2192.
21. Hughson EJ and Hopkins CR. Endocytic pathways in polarized Caco-2 cells: identification of an endosomal compartment accessible from both apical and basolateral surfaces. *J Cell Biol.* 1990 Feb;110(2):337-348.
22. Zaro JL and Shen WC. Quantitative comparison of membrane transduction and endocytosis of oligopeptides. *Biochemical and Biophysical Research Communications.* 2003;307(2):241-247.
23. Ryser HJ, Drummond I, Shen WC. The cellular uptake of horseradish peroxidase and its poly(lysine) conjugate by cultured fibroblasts is qualitatively similar despite a 900-fold difference in rate. *J Cell Physiol.* 1982 Oct;113(1):167-78.
24. Ritchie M, Tchistiakova L, Scott N. Implications of receptor-mediated endocytosis and intracellular trafficking dynamics in the development of antibody drug conjugates. *MAbs.* 2013 Jan-Feb;5(1):13-21.
25. Yang J, Chen H, Vlahov IR, Cheng JX, Low PS. Evaluation of disulfide reduction during receptor-mediated endocytosis by using FRET imaging. *Proc Natl Acad Sci USA.* 2006 Sep;103(37):13872-13877.
26. Austin CD, Wen X, Gazzard L, Nelson C, Scheller RH, et al. Oxidizing potential of endosomes and lysosomes limits intracellular cleavage of disulfide-based antibody-drug conjugates. *Proc Natl Acad Sci U S A.* 2005 Dec 13; 102(50): 17987-17992.
27. Trail P, 2013. Antibody Drug Conjugates as Cancer Therapeutics. *Antibodies.* 2013;2(1): 113-129.
28. Huan M, Zhang B, Teng Z, Cui H, Wang J, et al. In Vitro and In Vivo Antitumor Activity of a Novel pH-Activated Polymeric Drug Delivery System for Doxorubicin. *PLoS ONE.* 2012;7(9):44116-44211.
29. Hochuli E, Bannwarth W, Döbeli H, Gentz R, Stuber D, 1988. Genetic Approach to Facilitate Purification of Recombinant Proteins with a Novel Metal Chelate Adsorbent. *Nature Biotechnology.* 1988;6(11):1321-1325.
30. Wang E, Brown PS, Aroeti B, Chapin SJ, Mostov KE, et al. Apical and basolateral endocytic pathways of MDCK cells meet in acidic common endosomes distinct from a nearly-neutral apical recycling endosome. *Traffic.* 2000 Jun;1(6):480-493.
31. Wang Y, Chen YS, Zaro JL, Shen WC. Receptor-mediated activation of a proinsulin-transferrin fusion protein in hepatoma cells. *J Control Release.* 2011 Nov;155(3):386-392.
32. Seetharam B and Yammani RR. Cobalamin transport proteins and their cell-surface receptors. *Expert Rev Mol Med.* 2003 Jun;5(18):1-18.
33. June RK, Gogoi K, Eguchi A, Cui XS, Dowdy SF. Synthesis of a pH-Sensitive Nitrilotriacetic Linker to Peptide Transduction Domains To Enable Intracellular Delivery of Histidine Imidazole Ring-Containing Macromolecules. *Journal of the American Chemical Society.* 2010;132 (31):10680-10682.
34. Kristensen M, Nielsen HM. Cell-Penetrating Peptides as Carriers for Oral Delivery of Biopharmaceuticals. *Basic Clin Pharmacol Toxicol.* 2016 Feb;118(2):99-106.