

Oral absorption-promoting of Panax notoginseng saponins-loaded Nanoparticles modified with Thiolated trimethyl chitosan and Wheat germ agglutinin

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Abstract

Traditional Chinese medicine (TCM) oral preparations is the main means of disease treatment in TCM clinically, but the bioavailability of TCM active ingredients such as panax noteginseng saponins (PNS) is low due to its poor intestinal absorption. In this study, PNS nanoparticle modified with thiolated trimethyl chitosan (TMC-Cys) and wheat germ agglutinin (WGA) (PP-WT NPs) was prepared to promote the oral absorption of PNS effectively. In vitro studies demonstrated that PP-WT NPs delayed the release of PNS and had strong anti-enzyme activities against acids and digestive enzymes in the gastrointestinal environment. And it has good cell uptake and transport capacity in the Caco-2/HT29 co-cultured cell model. In vivo animal experiments showed that PP-WT NPs could penetrate the mucus layer barrier, and the effective permeability coefficients for R1, Rg1, and Rb1 in the small intestine were 1.68, 1.64, and 1.63 times than that of free PNS, respectively. It was predicted that panax notoginseng saponins-loaded nanoparticles modified with thiolated trimethyl chitosan and wheat germ agglutinin provide an attractive strategy for improving the oral absorption of PNS.

Introduction

Oral administration is the most popular route of administration¹⁻⁴. Unfortunately, the bioavailability of low permeability drugs is low due to its physicochemical properties and enzymatic degradation in the gastrointestinal tract⁵. At present, many Chinese medicinal components have been proved to have high activity in vitro experiments, but after oral administration, some Chinese medicinal active components, such as Panax noteginseng saponin have problems of poor intestinal absorption and low bioavailability, which affect the clinical efficacy and patients' medication compliance⁶. Therefore, it is particularly important to improve the intestinal absorption capacity and oral bioavailability of low permeability drugs⁷.

Panax notoginseng saponins is widely used to treat cardiovascular and cerebrovascular diseases, urinary diseases, digestive diseases and respiratory diseases⁸⁻¹¹. At present, PNS is taken as the traditional Chinese medicine preparation for treating CVD clinically, but the injection is still the main preparation of PNS. Xueshuantong Injection has been approved as an additional therapy for the treatment of cardiovascular and cerebrovascular diseases. And the potential toxicity caused by Chinese medicine injections in recent years has aroused concerns about safety. By contrast, oral medications offer significant advantages in improving patient safety and adherence. PNS exhibit very low oral bioavailability due to low intestinal permeability¹¹. Therefore, how to improve the oral absorption and bioavailability of PNS and the development of new dosage forms require further research.

Nanoparticles Coated with polymer materials has significantly improved the oral absorption of low-permeability drugs^{12, 13}. N- trimethyl chitosan (TMC) has good solubility, positively charged, and can interact with negatively charged lipids and mucus proteins in intestinal epithelial cells¹⁴, opening tight intercellular junction and promoting drug transport and absorption by cell bypass. However, the strong

electrostatic repulsion between positive charges of TMC molecules reduces the flexibility of molecular chain and weakens the penetration ability of mucus layer^{15, 16}. Thiolated polymer is a new adhesive material for mucous membrane. Under physiological conditions, it forms disulfide bonds with cysteine-rich areas of mucus protein, and it can closely bind to mucous membrane in the form of covalent bonds, which can prolong the retention time, increase the local drug concentration and promote drug absorption¹⁷. At the same time, thiolated trimethyl chitosan can also promote permeability by inhibiting the activities of tyrosine phosphatase and P- glycoprotein¹⁸.

The intestinal epithelium is considered to be a limiting barrier to drug absorption, primarily through transcellular and paracellular pathways. At present, new methods such as ligand-modified drugs to promote the endocytosis and absorption of drugs through intestinal epithelial cells have become an important direction of oral administration system research¹⁹. The ligand-modified drug particles specifically bind to receptors in the mucus layer or intestinal epithelial cells, and are subsequently pressed into the cell membrane to form vesicles for transport to the cells. As a ligand, lectin is nontoxic, immunogenic, resistant to acids in the digestive tract, and capable of degrading and destroying enzymes. It can also use the sugar residue of M cell in intestinal epithelial cells or Peyer's lymph node as a specific binding target. At present, tomato lectin (TL) and wheat germ lectin (WGA) have been used for the delivery of biological macromolecules such as protein and peptides. WGA-grafted nanoparticles were prepared and the association between Caco-2 monolayers and lipid nanoparticles indicated that WGA enhanced the binding and uptake of lipid nanoparticles to cells. This suggests that energy-dependent and sugar-specific interactions may help enhance the binding of WGA-grafted lipid nanoparticles to cells, triggering an endocytosis mechanism for their absorption²⁰.

To our best knowledge, both thiolated polymers and wheat germ agglutinin have been studied, however, there was no previous report on the two work together to enhance oral absorption. In this study, a novel oral drug delivery system based on the bioadhesion and permeation-promoting properties of cysteine and chitosan derivatives, as well as the WGA-mediated endocytosis of intestinal epithelial cells was designed (Fig. 8). The hydrophilic drug PNS with cardiovascular efficacy was selected as the model drug, and the nanoparticles of panax noteginseng saponins modified with TMC-Cys and WGA was prepared by emulsion-solvent evaporation method. The Caco-2/HT29 coculture cells and SD rats were used as experimental models to evaluate the drug-loaded nanoparticles, further validating that the drug delivery system synergistically absorbed the drugs through cell bypass transport and WGA-mediated endocytosis. The novel oral administration system constructed in this study effectively promoted the intestinal absorption of PNS. The work would pave a way for exploring the potential of low-permeability oral drugs.

Material And Methods

Material

TMC-Cys (free sulfhydryl content: 94.6% $\mu\text{mol/g}$) were prepared by our laboratory. PLGA (DG-50DLGH018) was purchased from Jinan Daigang Biotechnology Co., Ltd. WGA was purchased from Medicago. PNS(R1:8.9%, Rg1: 28.3%, and Rb1: 32.1%) was purchased from Chuxiong Yunzhi Pharmaceutical Co.,Ltd. 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC·HCL), N-hydroxysuccinimide (NHS), Colchicine, indomethacin and quercetin were purchased from Aladdin Co., Ltd. Ultra-filtration centrifugal tubes (cut-off of Mw 10 kDa, 100 kDa) were purchased from Millipore Co., Ltd. Dialysis tube (cut-off of Mw 8–14 kDa) was obtained from Spectrum Laboratories Co., Ltd. Pepsin (250 U/mg), trypsin (250 U/mg), DMSO (cell grade), PBS, PBST, HBSS, PMSF, MTT, DAPI, 4% tissue fixative, sodium azide, coumarin 6, and anti-fluorescence quencher were all purchased from Solarbio Science & Technology Co., Ltd. Fetal bovine serum (FBS), basal medium (MEM), non-essential amino acids, and sodium pyruvate were purchased from Gibco Technology Co., Ltd. The BCA protein concentration determination kit and RIPA cell lysis buffer were purchased from Beyotime Bio. Co., Ltd. Chlorpromazine was purchased from MedChemExpress.

Caco-2 and HT29 human colonic adenocarcinoma cells were purchased from the China Academy of Sciences cell bank. Female and male Sprague-Dawley (SD) rats (220 ± 20 g) were purchased from Slack Jingda Laboratory Animal Co., Hunan Province. All animal experiments are complied with the Guiding Principles for Animal Experiment of Guilin Medical University and approved by the animal ethics committee of the institution.

Preparation of Nanoparticles (NPs)

Preparation of Panax noteginseng saponins nanoparticle

Panax noteginseng saponins nanoparticles (PP NPs) were prepared by multiple emulsion solvent evaporation method. First, PLGA (50 mg) was dissolved in ethyl acetate, and Labrafim 1994 CS (40 μl) was added as oil phase. PNS (50 mg) was dissolved in PBS (pH 7.4, 0.01 M) as the internal aqueous phase. The internal aqueous phase was injected into the oil phase and vortexed, followed sonicated at 190 W for 2 min in an ice bath (5 off for 5 s) to obtain macroemulsion. The mixture was then transferred to 3 ml 1% PVA outer aqueous phase, sonicated at 190 W and vortexed sufficiently for 8 min (5 off for 5 s) to obtain W/O/W multiple emulsion dispersed in 12 ml 1% PVA. The suspension was stirred at low speed for 2 h at room temperature, removed organic solvent by rotary evaporation under reduced pressure at 30°C for 10 min, and dialyzed in a dialysis bag (cut-off Mw 8–14 kDa) for 4 h to remove free PNS. The upper layer was the PP NPs.

Surface modified nanoparticles

First, 1 ml of PP NPs was mixed with 1 ml of PBS (pH 7.4, 0.01 M) solution containing EDAC/NHS (3.5%/0.15%) and shaken gently for activation at room temperature for 2 h. After the suspension was placed in an ultrafiltration centrifuge tube (cut-off of Mw 10 kDa) and centrifuged at 4000 rpm for 30min, excess free reaction medium was removed, and then 0.8 ml of PBS(pH 7.4, 0.01 M) solution containing 0.2mg of wheat germ agglutinin (WGA) was added and incubated at 4°C for 16 h. Finally, the suspension

was centrifuged and placed in an ultrafiltration centrifuge tube (cut-off of Mw 100 kDa) for 30min at 4°C and 4000 rpm to remove the unreacted excess wheat germ lectin, and the nanoparticle solution in the filter cup was collected to obtain wheat germ lectin modified nanoparticles (PP-W NPs). The appropriate amount of TMC-Cys was added into the prepared nanoparticles, and stirred at low speed for 2 days to obtain TMC-Cys-adsorbed WGA-coupled panax noteginseng saponins nanoparticles (PP-WT NPs).

Cou-6 labeled PP/PP-W/PP-WT NPs (PP/PP-W/PP-WT NPs@Cou-6) were prepared by the above method. PNS was replaced with Cou-6, which was then dissolved with PLGA in ethyl acetate. The other steps are the same as for the preparation of PP/PP-W/PP-WT NPs.

Characterization of nanoparticles

Particle size and zeta potential

The particle size and Zeta potential of the prepared nanoparticles were determined by the particle size analyzer (Nano ZS90, Malver, UK). The prepared nanoparticles were diluted with pure water for a certain number of times, and the average value of the results of three consecutive experiments was taken as the final experimental result.

Morphological observation

The PP NPs and PP-WT NPs were appropriately diluted with purified water and then the sample dried on copper-coated grids for observation of morphologies by transmission electron microscopy (TEM, HT7700, Hitachi, Japan).

Encapsulation efficiency, drug loading capacity and WGA grafting ratio

A proper amount of methanol was added into the prepared nanoparticle suspension to destroy the nanostructures, and the contents of the three saponin components R1, Rg1 and Rb1 were determined by HPLC (Shimadzu, Japan). Determine the content of WGA with an ultraviolet spectrophotometer (INESA, China). The entrapment efficiency (EE %) and drug loading (DLC %) of the nanoparticles and the WGA grafting ratio can be calculated as follows ^{9,21}:

$$EE (\%) = \frac{\text{The quality of PNS in nanoparticles}}{\text{Total mass of PNS}} \times 100\%$$

$$DLC (\%) = \frac{\text{The quality of PNS in nanoparticles}}{\text{Total mass of nanoparticles}} \times 100\%$$

$$\text{Grafting ratio} (\%) = \frac{\text{Initial addition of WGA} - \text{Unbound WGA}}{\text{Initial addition of WGA}} \times 100\%$$

In vitro release studies

The in vitro release behavior of PNS in nanoparticles was determined by analytical bag method. A known strength suspension of PP-WT NPs was placed in 40 mL of dialysis medium (PBS, pH 7.4). Dialysis bag (cut-off Mw 8–14 kDa) with fine hemp rope fastened at both ends, to prevent leakage, shaken at 100 rpm in a shaker at 37°C. Samples (1 ml) were taken at predetermined intervals (0.5 h, 1 h, 1.5 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h, 24 h, 36 h, and 48 h) and the equal volume of fresh release solution at the same temperature was added. The released solutions taken out were filtered through a 0.45µm filter membrane, and then the PNS content was determined by HPLC, and the cumulative release percentage of the drug was calculated. The experiment were carried out triplicate.

Stability Assay in Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF)

PNS, PP-W NPs, and PP-WT NPs were placed in simulated gastric fluid (pH 1.2, HCl 1% (w/v) pepsin) and simulated intestinal fluid (pH 6.8, PBS with 1% (w/v) trypsin) in 37°C, 50 rpm water bath. 500µl samples of simulated gastric fluid were taken at (0, 15, 30, 60 and 120 minutes) and 1.0 ml methanol was added to stop the reaction. 500µl samples of artificial intestinal fluid were taken at (0, 15, 30, 60, 120 and 240 minutes) and 1.0 ml methanol was added to stop the reaction. The concentration of the drug was determined by HPLC. The experiment were carried out triplicate.

Cell studies in vitro

Caco-2 and HT29 cell culture

Human colon cancer Caco-2 and HT29 cells were purchased from the cell bank of the Committee for the Preservation of Type Culture, China Academy of Sciences. MEM containing 20% FBS, 1% non-essential amino acids, 1% sodium pyruvate and 1% streptomycin mixture (double antibody) was used for culture. HT29 cells can secrete mucus. Co-culture of Caco-2 and HT29 cells in a ratio of 9: 1 is the optimal ratio for permeability study of the in vitro model, and can better simulate human intestinal epithelial cells²².

Cytotoxicity evaluation

The cytotoxicity of the nanoparticles loaded with PNS was evaluated using the MTT assay. Approximate 1×10^4 Caco-2 and HT29 co-cultured cells were inoculated into 96 wells and cultured in 5% CO₂ at 37°C for 24 h. After the medium was aspirated, 100 µl of free PNS, PP NPs, PP-W NPs, and PP-WT NPs solutions in concentrations of (4.2 µg/ml, 8.3 µg/ml, 12.5 µg/ml, 16.7 µg/ml, and 25 µg/ml) were added, respectively. After incubation for 24 h, 20 µl of MTT solution was added to each well and incubated in the dark at 37°C for 4 h. The supernatant was removed, added 150µl of dimethylsulfoxide to each well, and shaken the solution at low speed for 10 min. After the violet crystals were completely dissolved, measured the absorbance (OD) at a wavelength of 490 nm.

Using the same method, 100 µl of free Cou-6 solution at concentrations of (25 ng/ml, 50 ng/ml, 75 ng/ml, 100 ng/ml, and 150 ng/ml), PP NPs@Cou-6, PP-NPs@Cou-6, and PP-WT NPs@Cou-6, respectively, were added to calculate the cytotoxic effect of the loaded Cou-6 nanoparticles on cells.

$$\text{Cell viability}\% = \frac{OD_{\text{Dosing}} - OD_{\text{blank}}}{OD_{\text{Control}} - OD_{\text{blank}}} \times 100\%$$

Cellular uptake assay

Qualitative and quantitative assessment of cellular uptake can be performed to investigate drug uptake in the intestine²³. The Caco-2 and HT29 co-cultured cells were incubated with Cou-6 solution (100 ng per well), PP-NPs@Cou-6, PP-W NPs@Cou-6, and PP-WT NPs@Cou-6 at the same drug concentration in the dark for 3 h at 37°C, followed by gentle washing with cold PBS (pH 7.4) to stop uptake. For qualitative uptake, the samples were successively fixed with 4% paraformaldehyde for 15 min, washed with PBS for 3 times, the nuclei were stained with DAPI for 8 min, washed with PBST for 3 times, and then washed with PBS for 3 times. The cell slides were taken out and sealed with an anti-fluorescence quencher, and drug uptake on the cell slides was observed under an upright fluorescence microscope (Carl Zeiss AG, Zeiss Axio Imager Z2). For quantitative uptake, 100 µl cell lysates were added to each well, lysed at 4°C for 30min in the dark, and then centrifuged at 12000 rpm at 4°C for 30 min to obtain the supernatant. They were placed in a microplate reader to determine fluorescence intensity (Ex = 466 nm, Em = 504 nm) and protein concentration in the cells by BCA method.

Cellular uptake mechanism

Caco-2 and HT29 co-cultured cells were inoculated in 96-well plates at a density of 1×10^4 . After 24 h of culture, inhibitors (including the omental inhibitor colchicine 5 µg/ml, the ATP inhibitor sodium azide 3 mg/ml, the reticulon inhibitor chlorpromazine 10 µg/ml, the caveolin inhibitor indomethacin 200 µmol/ml, and the non-reticulon non-caveolin inhibitor quercetin 10 µmol/l) were added. Inhibitors were removed after 2 h of incubation with cells and washed 3 times with PBS. 100 µl of 100 ng/ml PP-WT NPs@Cou-6 was added to each well and incubated in the dark at 37°C for 2 h. After 30 minutes of lysis on ice with 150µl DMSO and complete lysis, wells were placed in a microtiter plate reader to determine fluorescence intensity (Ex = 466 nm, Em = 504 nm). The inhibition by each group of inhibitor was expressed as relative fluorescence intensity.

$$\text{Relative fluorescence intensity} = \frac{A_{\text{inhibitiongroup}} - A_{\text{blankgroup}}}{A_{\text{controlgroup}} - A_{\text{blankgroup}}} \times 100\%$$

Transport through Caco-2&HT29 coculture cell monolayers

The Caco-2 and HT29 coculture cells were inoculated in 3×10^4 /well on the AP side of 24-well transwell(0.4 µm, growth area of 0.33 cm²), and 800µl of complete culture medium was added for BL measurement. The transmembrane resistance (TEER, Ω·cm²) between monolayers of Caco-2&HT29 co-cultured cells was measured using the Millicell ERS-2 system on days 2, 3, 5, 7, 9, 12, 15, 18, 20, and 21. The TEER value above 250 Ω·cm² indicated that the cell monolayer could be used for transmembrane experiment.

Before the experiment, the AP side and BL side of the chamber were gently rinsed with HBSS for three times, then an appropriate amount of HBSS was added, the cells were equilibrated at 37°C for 30 min, and HBSS was sucked and discarded. On the AP side, 0.3 ml of PP NPs@Cou-6, PP-W NPs@Cou-6, and PP-WT NPs@Cou-6 (Cou-6 concentration 500 ng/ml) were added respectively, and on the BL side, 1 ml HBSS were added. At various intervals (0.5, 1, 1.5, 2, and 3 h), 0.2 ml of sample was taken from the BL side and 0.2 ml of isothermal HBSS was replenished. After incubation for 3 h, the nanoparticle solution was removed, rinsed gently with HBSS and incubated in medium for an additional 24 h and the TEER values of the cell monolayers were determined at intervals (0.5, 1, 1.5, 2, 3, 6, 10, and 24 h). The samples were placed in a microplate reader to determine fluorescence intensity and the apparent permeability coefficient (P_{app} , cm/s) of Cou-6 was calculated as follows:

$$P_{app} = \frac{Q}{A * C * T}$$

Q: Cou-6 cumulative permeability (ng)

A: surface area of cell monolayers (cm²)

C: Initial concentration (ng/cm³) of Cou-6 in the apical side

T: incubation time

Animal evaluation

Permeability evaluation of mucus layer

According to the literature method, the mucus was collected within 2 h after the SD rats were sacrificed^{24, 25}. After the intestine was rinsed with pre-cooled HBSS, the mucus was collected by gently scraping with a spatula and stored at -80°C for subsequent use. The permeability of modified NPs through the mucus layer was studied using a Transwell system²⁶. A total of 100 µl intestinal mucus was uniformly placed on the top side of Transwell chamber, and 800 µl HBSS solution was added and equilibrated on the bottom side for 15 min. Then add 200 µl of PP NPs@Cou-6, PP-W NPs@Cou-6 and PP-WT NPs@Cou-6 on the top side (Cou-6 concentration: 500 ng/ml). The chamber was held at 37°C and the sample was removed 200 µl from the bottom and replenished with 200 µl of fresh HBSS solution at 15, 30, 60, 90, and 120 min. Each sample was measured using a fluoroenzyme microplate reader (Ex = 466 nm, Em = 504 nm) and the cumulative permeability and apparent permeability coefficient of Cou-6 were calculated.

Permeability of isolated small intestine

The intestinal permeability of nanoparticles before and after modification was studied using the intestinal permeability junction model. Duodenum, jejunum and ileum (5 cm) of SD rats were taken and ligated at both ends. 0.4 ml of PP NPs@Cou-6, PP-W NPs@Cou-6, and PP-WT NPs@Cou-6 (Cou-6 concentration: 500 ng/ml) were added to the intestinal lumen, respectively. The bowel segments were

placed in conical flasks containing 10 ml of oxygenated Krebs-Ringer buffer (K-R buffer) at 37°C and shaken at 100 rpm. At 0.5, 1, 1.5, and 2 h, 200 µl samples were taken out and added an equal volume of fresh K-R buffer. The determination method of Cou-6 was the same as above.

Observation of intestinal villus absorption

The in vivo intestinal ligation model was used to study the villi absorption of nanoparticles in different intestinal segments. The SD rats were randomly divided into five groups. In the experiment, 2% pentobarbital sodium was injected into the abdominal cavity for anesthesia, and the abdominal cavity was opened. The duodenum, jejunum and ileum, approximately 2 cm in length, were ligated with surgical wires. Then, 0.1 ml of Cou-6 Sol, PP NPs@Cou-6, PP-W NPs@Cou-6, and PP-WT NPs@Cou-6 (each containing Cou-6 100 ng) were injected into the intestinal lumen of each of the four groups of rats. The rats in the other group were injected with 0.1 ml normal saline into the intestinal lumen. The intestinal segments were taken out after 2 h, rinsed with PBS buffer pre-cooled at 4°C and then put into OCT embedding medium for frozen sections. The sections were fixed with 4% paraformaldehyde for 10 minutes, stained with DAPI, and fixed with an anti-fluorescence quencher. The samples were placed under an upright fluorescence microscope(Carl Zeiss AG, Zeiss Axio Imager Z2) to observe the absorption of nanoparticles in the villi of different intestinal segments²⁷.

In vivo intestinal recirculation perfusion

SD rats (Male and female) were fasted (without water) for 12 h prior to the experiment. Animals were anesthetized by intraperitoneal injection of 2% pentobarbital sodium and fixed on the back. The abdominal cavity was opened, and incisions were made at the upper end of the duodenum and the lower end of the ileum, respectively. A glass tube with a diameter of about 0.5 cm was inserted, and then ligated with a surgical wire. The intestinal contents were drained with 37°C normal saline, and the glass tubes at both ends of the intestinal tube were connected with the peristaltic pump hose to form a ring after the intestinal tube was cleaned. The wound was moistened with gauze impregnated with normal saline at 37°C. The peristaltic pump hose was connected to the blank K-R buffer, and the peristaltic pump was started to prime at a flow rate of 2.5 ml/min for 30min to equilibrate the intestinal environment. The liquid was emptied and connected the solution containing the drug. Time from the time point at which the first drip flow was zero in the flask, the sample (2 ml) were removed at (0, 0.25, 0.5, 0.75, 1.5, 1.75, 2.0 h) from the infusion flask and added with equal volume blank phenol red solution. The concentration of PNS was measured by HPLC.

After the experiment, the rats were sacrificed and the small intestinal area of each rat was measured (A)²⁸. The calculation formulas of the absorption rate constant (K_a), absorption half-life ($t_{1/2}$), percent absorbed per unit time (P%), intestinal apparent permeability coefficient (p_{eff}) were as follows:

$$K_a = \frac{\ln(X_o/X)}{t}$$

$$t_{1/2} = \frac{0.693}{K_a}$$

$$P\% = CoVo - CnVn - V(Co + C1 + \dots + Cn - 1)/Co \times Vo \times T \times 100\%$$

$$P_{eff} = Ka/A/3600$$

$$A = 2\pi rL$$

r: cross-sectional area of the intestine in the perfusion section; L: The length of intestine in perfusion section; Xo: Initial drug amount of intestinal circulation liquid; X: Residual drug amount of intestinal circulating fluid; Co: initial concentration of liquid medicine; Vo: Initial volume of liquid medicine; Cn: concentration of the nth perfusion solution;

Vn: Volume of the nth perfusion liquid; T: cycle time; V: Volume per sample

Intestinal absorption mechanism

The intestinal absorption mechanism of nanoparticles was studied using an everted intestine model. SD rats were deprived of food and water for 12 h before the experiment. The rat was anesthetized by intraperitoneal injection of 2% pentobarbital sodium. The abdominal cavity was opened, and the duodenal segment was taken out. The intestinal segment was washed with K-R buffer preheated at 37°C and then everted. The intestinal segments were cut into small sections of approximately 60 mg and placed in 24-well cell culture plates. Each group of inhibitors (10 µM colchicine, 25 µM sodium azide, 50 µM chlorpromazine, 100 µM indometacin, 10 µM quercetin) was added to well plates at 37°C, and shaken at 50 rpm for 45 min. The inhibitor solution were removed, washed with warm K-R buffer, added 1 ml of PP NPs@Cou-6, PP-W NPs@Cou-6, and PP-WT NPs@Cou-6, respectively (200 ng/ml), and continue incubated at 37°C for 1.5 h. The sections without inhibitor at 37°C were the positive controls, and the sections without inhibitor at 4°C were the negative controls. After incubation, the drug solution was taken out, and 4°C pre-cooled K-R buffer was added into each well for washing. Finally, the bowel segments were transferred to a homogenate tube and 300 µl of K-R buffer was aspirated for homogenization. Then 100 µl samples were taken out and the content was determined by Fluorescent microplate reader.

Statistical analysis

All experimental data were expressed as mean ± standard deviation (SD). Analysis of variance was used for multi-group comparison. P-values less than 0.05 were considered statistically significant.

Results And Discussion

Preparation and characterization of nanoparticles

Figure 1 was illustrated the preparation process of nanoparticles. The PP NPs was prepared by emulsion-solvent evaporation method. The PP NPs was based carbodiimide bifunctional reagent to bind the

carboxyl on the surface of the nanoparticle carrier material with the amino group of wheat germ agglutinin molecule, and then utilized the electrostatic adsorption to bind PP-W NPs with negative charge and TMC-Cys with positive charge to prepare PP-WT NPs. As shown in Table 1 and Fig. 2, the particle size of the PP-WT NPs was increased from 208.3 ± 2.71 nm to 315.1 ± 4.98 nm, and the Zeta potential changed from -18.77 ± 1.95 mV to $+12.05 \pm 1.43$ mV. This was due to the positively charged amino group in both target WGA and TMC-Cys^{14,21}. It could be seen that both WGA and TMC-Cys were modified on the surface of the nanoparticles.

Table 1

Physical properties of PNS-loaded nanoparticle formulations (Data are presented as mean ± SD, n = 3)

Formulation	Size (nm)	PDI	Zeta potential (mV)	EE(100%)			DL(100%)	WGA grafting ratio(100%)
				R1	Rg1	Rb1		
PP NPs	208.3 ± 2.71	0.234 ± 0.012	-18.77 ± 1.95	49.65 ± 0.38	59.60 ± 1.18	73.72 ± 0.64	11.87 ± 0.23	—
PP-W NPs	252.6 ± 2.79	0.287 ± 0.011	-8.24 ± 1.21	29.06 ± 1.19	29.06 ± 1.19	30.97 ± 1.54	6.96 ± 0.12	68.03 ± 0.09
PP-WT NPs	315.1 ± 4.98	0.324 ± 0.024	+12.05 ± 1.43	34.90 ± 2.67	41.42 ± 1.74	36.59 ± 0.64	8.24 ± 0.35	67.92 ± 0.05

TEM images of PP NPs and PP-WT NPs, respectively, were shown in Fig. 2C and D. Distinctly, the modified PP-WT NPs exhibited a larger particle size than PP NPs, which was consistent with the results determined by the particle size analyzer.

In vitro release and stability assay

The in vitro release of PNS from PP-WT NPs in PBS was analyzed using a dialysis bag method. As shown in Fig. 2, from the release curve, R1 and Rg1 released more quickly, and Rb1 released more slowly. 60.15% of R1, 69.15% of Rg1, and 29.85% of Rb1 were released within the first 12 h. Obviously, if the drug is released in the gastrointestinal tract, it will be evacuated, and it is speculated that the nanoparticles are mainly absorbed into the cells of the gastrointestinal tract in an integrated form to release the drug into the blood circulation.

PNS is unstable and degrade rapidly in the gastrointestinal environment²⁹. The degradation rate of the drug under acidic conditions and exposure to digestive enzymes was studied. As shown in Fig. 3, after the drug substance of free PNS was cultured in simulated gastric fluid for 2 hours, the residual percentages of PNS were 37.90% for R1, 41.40% for Rg1 and 48.11% for Rb1, respectively. And after the drug substance of free PNS was cultured in simulated intestinal fluid for 4 hours, the residual percentages of PNS were 47.62% for R1, 86.93% for Rg1 and 77.09% for Rb1, respectively. Specifically for

PP-WT NPs, the residual percentages of encapsulated PNS were 46.26% for R1, 52.09% for Rg1, and 62.18% for Rb1 after 2 h of incubation in simulated gastric fluid, while 85.66% for R1, 88.44% for Rg1, and 83.28% for Rg1 after 4 hours of incubation in simulated intestinal fluid. The above results indicated that PP-WT NPs could protect effectively from the degradation of PNS in the gastrointestinal acid and enzymes.

Cell studies in vitro

In vitro cytotoxicity assay

To evaluate the cytotoxicity of PNS and Cou-6 nanoparticles, the cell viability of Caco-2&HT29 co-cultured cells with different concentrations of nanoparticles were determined by the method of MTT cell viability. As shown in Fig. 4A, when the concentration of PNS drug was in the range of 4.2–25 µg/ml, the cell viability of drug substances PNS, PP NPs, PP-W NPs and PP-WT NPs after cell treatment were above 94%, and almost no cell death was caused. Indicating that it was safe and harmless to cells within this concentration range. As shown in Fig. 4B, the cell-treated cell viability of Cou-6 solution, PP NPs@Cou-6, PP-W NPs@Cou-6, and PP-WT NPs@Cou-6 were above 91% over the range of 25 to 100 ng/ml for Cou-6, while the cell-treated cell viability of PP-W NPs@Cou-6 and PP-WT NPs@Cou-6 decreased to approximately 86% and 77% over the range of 150 ng/ml for Cou-6. The above results indicated that the concentration of Cou-6 was in a concentration-dependent manner with the cell viability, which was more reliable for the results of subsequent cell uptake experiments loaded with Cou-6 nanoparticles. Therefore, 100 ng/ml was selected as the dosing concentration of Cou-6.

In Vitro Cell Uptake Mechanism Assay

As shown in Fig. 4, the cellular uptake of Cou-6, PP NPs@Cou-6, PP-W NPs@Cou-6 and PP-WT NPs@Cou-6 in Caco-2&HT29 coculture were analyzed qualitatively and quantitatively. The qualitative results of cell uptake were shown in Fig. 4C. Both PP-W NPs@Cou-6 and PP-WT NPs@Cou-6 have higher green fluorescence intensity and stronger cell uptake capacity than unmodified PP NPs@Cou-6, indicating that the nanoparticles modified by WGA and TMC-cys effectively promoted the uptake of drugs into cells. The quantitative results of cellular uptake were shown in Fig. 4D, where the cellular uptake of the drug in PP-W NPs@Cou-6 and PP-WT NPs@Cou-6 were 1.51 times and 1.78 times higher, respectively, than in PP NPs@Cou-6. It could be seen from the experimental results that the nanoparticles synergistically modified by WGA and TMC-Cys could enhance the drug uptake ability of cells, and this experimental result was consistent with the qualitative experimental result. At the same time, the above experimental results further verified that the penetration-promoting effect of TMC in trimethyl chitosan derivatives and the mucosal adhesion in cysteine reported in the literature played a certain role in cell uptake³⁰, and wheat germ lectin WGA mediated the endocytosis of intestinal epithelial cells³¹. Both have the effect of synergistically promoting oral absorption of the drug.

To further clarify the uptake pathway of nanoparticles, the five endocytosis inhibitors were used in the present study to investigate the uptake of PP-WT NPs@Cou-6 in Caco-2&HT29 coculture cells. As shown in Fig. 4E, the uptake of nanoparticles by cells treated with NaN_3 (ATP ase inhibitor) was 86.23% of that in the control group, indicating that the uptake of nanoparticles by cells required energy consumption. Among that endocytosis inhibitor studied, only the colchicine-treated cell showed statistically significant differences in the uptake of nanoparticles, which was 76.03% of that in the control group. Cell uptake mechanism experiment showed that PP-WT NPs entered cells mainly through the giant pinocytosis pathway.

Transport through Caco-2&HT29 coculture cell monolayers

The results of transport through Caco-2&HT29 coculture cell monolayers were shown in Fig. 5. It was reported that when the TEER value was greater than $250 \Omega \cdot \text{cm}^2$, the permeability of the drug could be measured using Caco-2&HT29 cell monolayers³². As shown in Fig. 5B, after 21 days of culture, the TEER value was above $250 \Omega \cdot \text{cm}^2$, indicating that the monolayers of Caco-2&HT29 cells were successfully constructed³³. The penetration into Caco-2&HT29 cell monolayers indicated that the transport of PP-W NPs@Cou-6 and PP-WT NPs@Cou-6 increased significantly with the prolongation of culture time, which were higher than those of PP NPs@Cou-6 (Fig. 5D). The P_{app} values of PP-W NPs@Cou-6 and PP-WT NPs@Cou-6 were 2.04 and 1.28 times, respectively, higher than those of PP NPs@Cou-6. It was further confirmed that the WGA-modified nanoparticles could enhance the drug transport through the Caco-2&HT29 cell monolayers (Fig. 5E). Interestingly, the cumulative permeability of PP-W NPs@Cou-6 was higher than that of PP-WT NPs@Cou-6, suggesting that wheat germ agglutinin and TMC-Cys had competitive inhibition on this cell monolayer, leading to better drug transport results of the nanoparticles modified with wheat germ agglutinin in the cell monolayer. Figure 5C shows that the TEER value of Caco-2&HT29 cell monolayers treated with PP-WT NPs@Cou-6 was decreased to approximately 57%. After removal of the PP-WT NPs@Cou-6 solution, the TEER values was gradually increased and returned to their original levels. However, there were no significant changes in TEER values for Caco-2&HT29 cell monolayers in the untreated and other experimental groups (PP NPs@Cou-6 and PP-W NPs@Cou-6). These phenomena suggest that PP-WT NPs could enhance the paracellular transport of drugs by reversibly opening a tight junction. It could be preliminarily speculated that TMC-Cys played a role, which was consistent with the reported role of chitosan in reversibly opening the tight junction between cells¹⁴.

Animal evaluation

Permeability evaluation of mucus layer

In the Transwell chambers containing rat intestinal mucus, the cumulative permeability and apparent permeability P_{app} values of Cou-6 loaded with different Cou-6 nanoparticles were shown in Figs. 6A and B, respectively. After 1 h, the cumulative permeability of Cou-6 through the mucus layer was higher for PP-WT NPs@Cou-6 and PP-W NPs@Cou-6, respectively, while that of PP-WT NPs@Cou-6 was slightly higher

than that of PP-W NPs@Cou-6. At 2 h, the apparent permeability P_{app} of PP-W NPs@Cou-6 and PP-WT NPs@Cou-6 was $(14.15 \pm 0.12) \times 10^{-6} \text{ cm} \times \text{s}^{-1}$ and $(15.60 \pm 0.29) \times 10^{-6} \text{ cm} \times \text{s}^{-1}$, respectively, which was 1.49 and 1.65 times the apparent permeability value of PP NPs@Cou-6. The results showed that the PP-WT NPs could effectively promote the drug permeation through the mucus layer barrier.

Permeability of isolated small intestine

The permeation effects of PP NPs@Cou-6, PP-W NPs@Cou-6 and PP-WT NPs@Cou-6 into the isolated small intestine were analyzed by using a fluorescent quantitative microplate reader, as shown in Figs. 6C, D and E. Drug penetration was time-dependent and increased with time. At 2 h of comparison, the cumulative permeability of PP-W NPs was higher than that of PP NPs in the duodenum and jejunum, but PP-W NPs was slightly lower than that of PP NPs in the ileum. When incubated at the same intervals in the same intestinal segment, the cumulative permeability of PP-WT NPs was higher than both PP-W NPs and PP NPs. These results indicated that WGA played an important role in mediating the endocytosis of intestinal epithelial cells. TMC-Cys could provide a strong mucosal adhesion and increase the permeability of drugs in the mucus layer. Combined with the mucus layer permeability evaluation results, PP-WT NPs could be able to provide strong mucoadhesion and increase the permeability of drug in the mucus layer and small intestine.

Observation of intestinal villus absorption

In order to better verify the absorption of PP-WT NPs in the small intestine, frozen sections of tissues from different intestinal segments (duodenum, jejunum and ileum) were observed under an orthostatic fluorescence microscope as shown in Figs. 7A, B and C. In the three different intestinal segments, the green fluorescence of Cou-6 in the absorption of small intestinal villi after treatment with PP-WT NPs@Cou-6 was significantly higher than that in Cou-6 solution, PP NPs@Cou-6 and PP-W NPs@Cou-6, and the intensity of green fluorescence entering the intestinal intima of drugs in each group was PP-WT NPs > PP-W NPs > PP NPs. It indicated that PP-WT NPs could enhance the penetration of drugs through the mucus layer and into the inner membrane of the small intestine, thereby improving the ability of drugs to be absorbed by the small intestine. It verified that TMC-Cys was promoted the penetration of drugs through the mucus layer and enhanced the permeability of small intestinal epithelial cells. While WGA was targeted to enhance the endocytosis of drugs in small intestinal villous epithelial cells. The two synergistically were played the role of drugs penetration through the mucus layer and into small intestinal epithelial cells, thereby enhancing the ability of drugs to be absorbed by the small intestine.

In vivo intestinal recirculation perfusion

In the intestinal recirculation experiment, the absorption parameter of small intestine was calculated by determining the residual amount of drugs in the enema. This method excludes the first-pass effect of the liver and the effect of gastric contents on the experiment, so the measured data could be the actual reaction of drug absorption characteristics in the small intestine. Table 2 and Table 3 show that the K_a values of the three components of PP-WT NPs were greater than that of the PNS solution, while the PP

NPs were slightly lower than that of the PNS solution, demonstrating that the absorbance of the TMC-Cys and WGA modified nanoparticles were greater than that of the PNS drug substance. The decrease in $t_{1/2}$ of the three components in PP-WT NPs also indicates that the absorption rate of nanoparticle were more rapidly. Linear regression of the logarithm of the residual drug amount ($\ln X$) to time (t) was performed and the results was a straight lines, indicating that the PNS nanoparticles were in accordance with the first-order elimination kinetics. Table 5 shows the P_{eff} values of the three components in the PNS drug substance were 0.82×10^{-6} , indicating that the absorption of PNS drug substance was poor. The P_{eff} values of the three components of PP-WT NPs were all greater than 1.20×10^{-6} , indicating that the modified nanoparticles could improve the absorption effect of PNS and the components R1, Rg1 and Rb1 had been converted from hard-to-absorb components to easy-to-absorb components.

Table 2

$K_a(h^{-1})$ values of each component of different preparations
(Data are presented as mean $\pm$ SD, n = 6).

Formulation	R1	Rg1	Rb1
PNS	0.20 ± 0.06	0.19 ± 0.03	0.26 ± 0.03
PP NPs	0.18 ± 0.08	0.15 ± 0.05	0.22 ± 0.06
PP-WT NPs	$0.31 \pm 0.08^*$	$0.29 \pm 0.08^*$	$0.38 \pm 0.06^{**}$

Table 3

$t_{1/2}(h)$ values of each component of different preparations
(Data are presented as mean $\pm$ SD, n = 6).

Formulation	R1	Rg1	Rb1
PNS	3.84 ± 1.41	3.70 ± 0.63	2.66 ± 0.32
PP NPs	5.08 ± 3.19	5.08 ± 2.07	3.46 ± 1.57
PP-WT NPs	2.38 ± 0.67	2.59 ± 0.87	1.86 ± 0.28

Table 4

$P\%(h^{-1})$ values of the components in different preparations
(Data are presented as mean $\pm$ SD, n = 6).

Formulation	R1	Rg1	Rb1
PNS	0.04 ± 0.02	0.04 ± 0.03	0.09 ± 0.04
PP NPs	0.05 ± 0.04	0.03 ± 0.02	0.08 ± 0.03
PP-WT NPs	$0.15 \pm 0.08^{**}$	$0.11 \pm 0.06^*$	$0.22 \pm 0.04^{****}$

Table 5

P_{eff} ($\times 10^{-6} \times \text{cm} \times \text{s}^{-1}$) values of each component of the different preparations (Data are presented as mean $\pm$ SD, $n = 6$).

Formulation	R1	Rg1	Rb1
PNS	0.76 ± 0.27	0.73 ± 0.18	0.98 ± 0.23
PP NPs	0.57 ± 0.27	0.51 ± 0.07	0.73 ± 0.20
PP-WT NPs	$1.28 \pm 0.22^{**}$	$1.20 \pm 0.31^{**}$	$1.60 \pm 0.28^{***}$

Intestinal absorption mechanism

To further clarify the absorption pathway of PP-WT NPs, the intestinal absorption mechanism was studied *in vivo* (Fig. 8). The intestinal absorption of nanoparticles requires energy and a suitable temperature. As shown in Figs. 8A, B, and C, the uptake of PP-W NPs Cou-6 in the colchicine-treated small intestine was 75.88% of that in the control group, with no significant inhibition by other inhibitors. These results indicated that PP-W NPs could only be absorbed by megakaryocyte. The uptake of PP-WT NPs after treatment with the four inhibitors (colchicine, chlorpromazine, indomethacin, and quercetin) were 79.43%, 80.65%, 63.65%, and 60.59% of that in the control group, respectively. All of this indicates that the intestinal absorption of PP-WT NPs involves megakaryocyte, reticulin, caveolin, non-reticulin and non-caveolin-mediated endocytosis. Therefore, compared with unmodified PP NPs, PP-WT NPs can enter cells through more endocytosis pathways to promote intestinal absorption. However, this result was not consistent with the experimental results on the uptake mechanism of PP-WT NPs@Cou-6 in adherent cells, which might be due to the fact that absorption in the body and intestine was a complex regulatory process, which includes not only mucosal endocytosis, but also intracellular transport of the carrier and serosal exocytosis.

Conclusion

In this study, PNS nanoparticles modified with trimethyl chitosan derivative (TMC-Cys) and wheat germ agglutinin were successfully prepared and characterized by physicochemical properties. Cell and animal experiments showed that PP-WT NPs could promote the oral absorption of PNS based on the bioadhesion and permeability-promoting characteristics of cysteine and chitosan derivatives, as well as WGA-mediated endocytosis of intestinal epithelial cells. The results demonstrated that PP-WT NPs were a potential oral delivery of the BCS class III drugs platform for improving the intestinal absorption of drug.

Declarations

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Author contribution

Ruiyue Fang: Conducting experiments, data analysis, investigation, and writing original draft; Yue Wei, Shiyuan Lin, Ying Zhao: Data analysis; Hui Chen: Experimental design, interpretation of data, and project administration.

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Availability of data and materials

The data that support the findings of this study are available from the author, Ruiyue Fang, upon reasonable request.

Ethical approval

All institutional and national guidelines for the care and use of laboratory animals were followed.

Consent to participate

Written informed consent was obtained from participants for publication of this manuscript.

Consent for publication

Written informed consent was obtained from the authors for publication of this manuscript.

Competing interests

No potential conflict of interest was reported by the authors.

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Figures

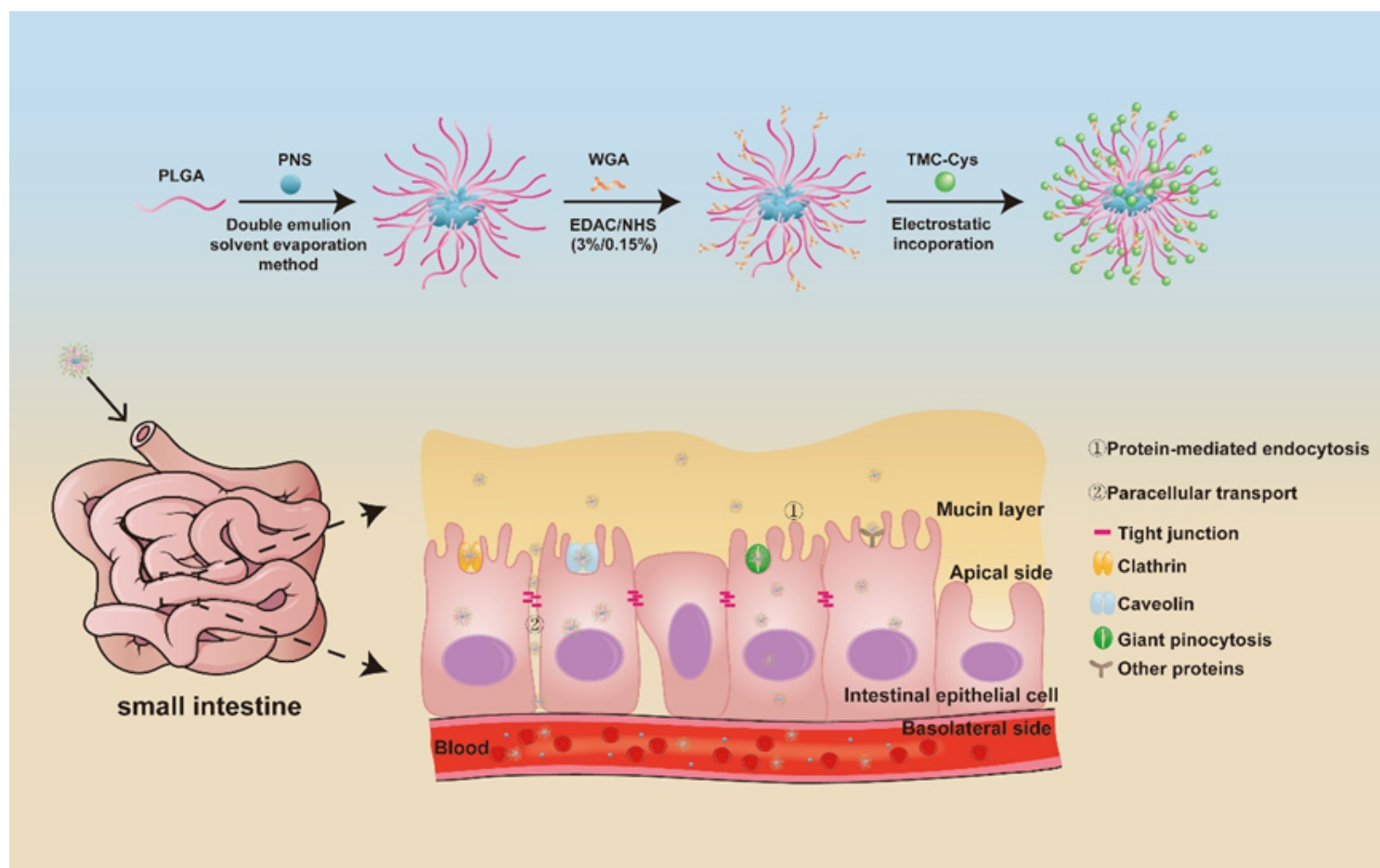


Figure 1

The enhanced effect of PP-WT NPs on the oral absorption of PNS.

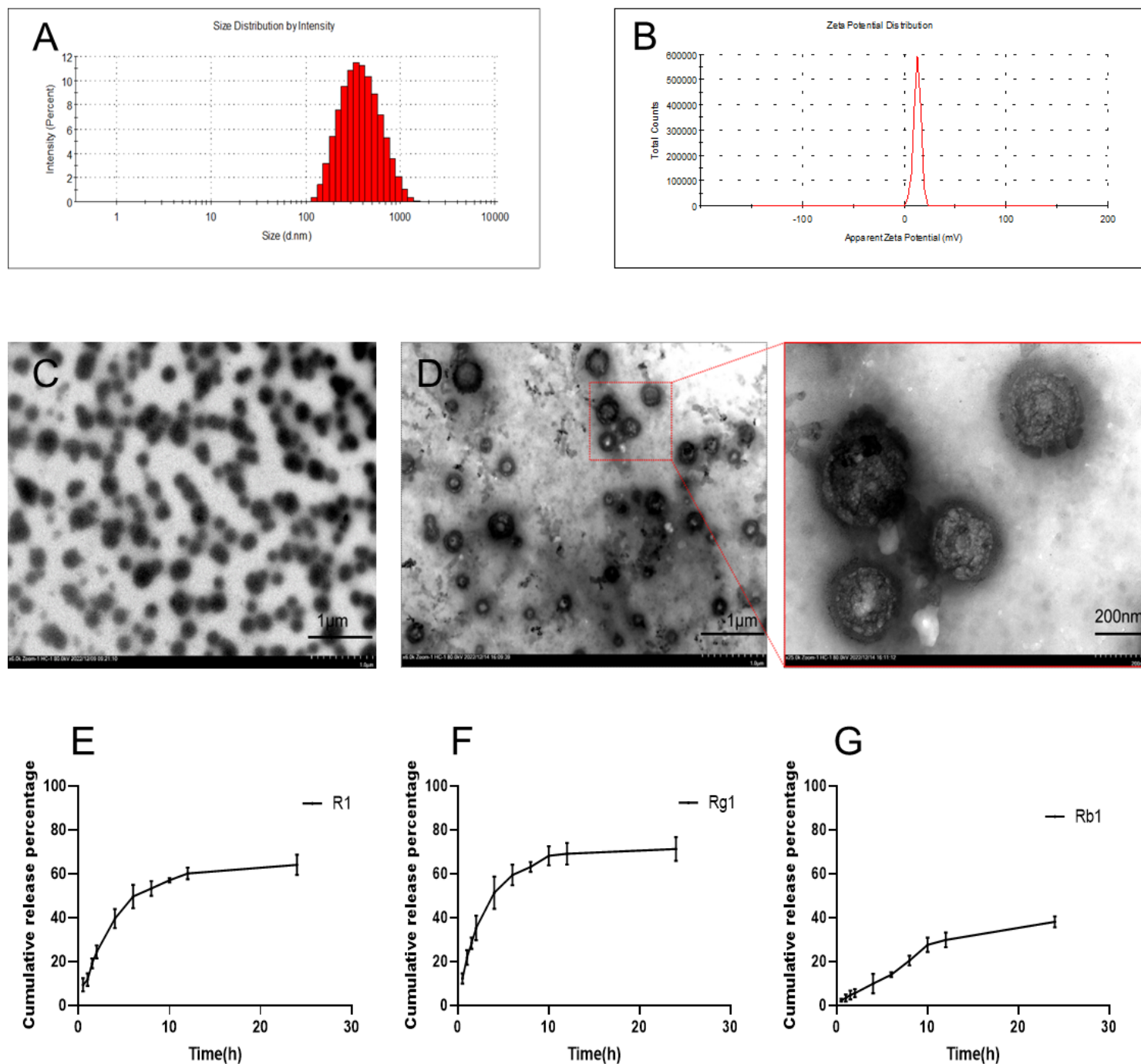


Figure 2

Particle size (A) and potential (B) of PP-WT NPs. The TEM images of PP NPs (C) and PP-WT NPs (D). In vitro release profile of PP-WT NPs in PBS (E: R1, F: Rg1, G: Rb1). Data are presented as mean $\pm$ SD (n = 3).

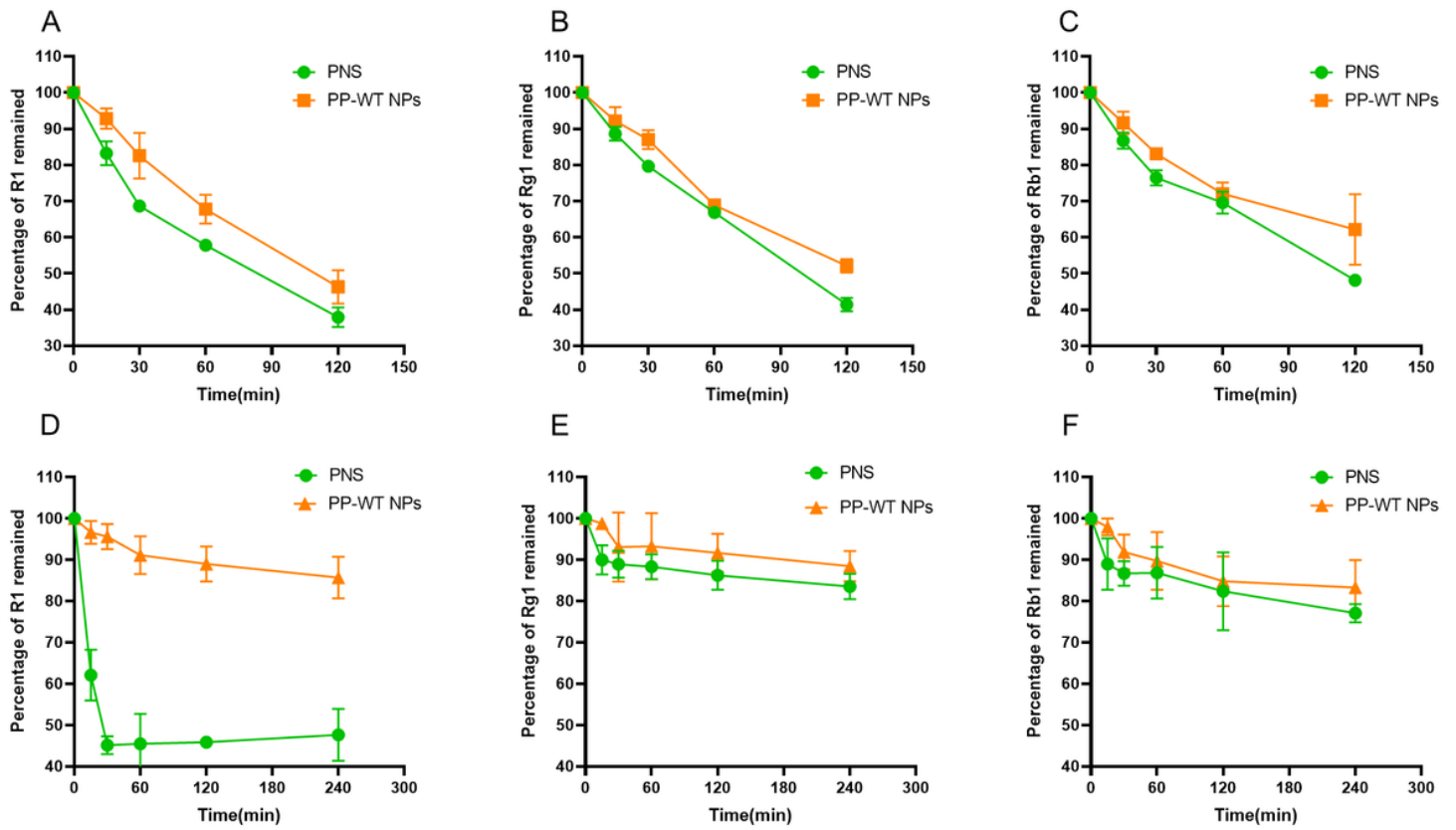


Figure 3

Percentage of R1 (A), Rg1 (B), Rb1 (C) remained after incubation in SGF and percentage of R1 (D), Rg1 (E), Rb1 (F) remained after incubation in SIF. Data are presented as mean $\pm$ SD (n = 3).

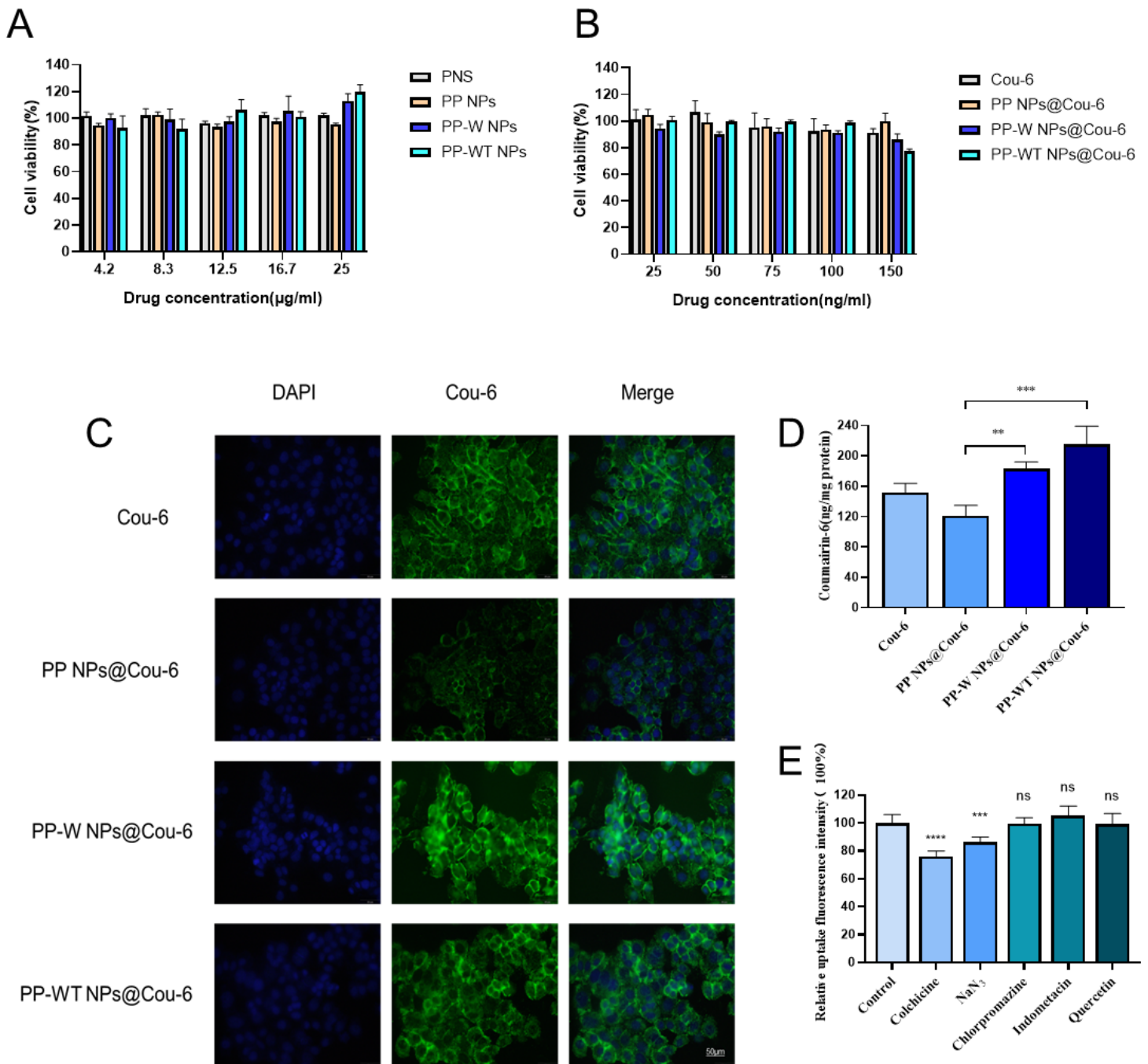


Figure 4

Cell viability of PNS nanoparticles and PNS (A)/ Cou-6 nanoparticles and Cou-6 (B) at different concentrations. Data are presented as mean $\pm$ SD (n = 5). (C) The qualitative and (D) the quantitative uptake of Cou-6, PP NPs@Cou-6, PP-W NPs@Cou-6, PP-WT NPs@Cou-6 in Caco-2&HT29 coculture cells. (E) The effect of different endocytosis inhibitors on the uptake of PP-WT NPs@Cou-6 in Caco-2&HT29 coculture cells. Data are presented as mean $\pm$ SD, **p<0.01, ***P<0.001, ****P< 0.0001, no significant trend in ns P values (n = 3).

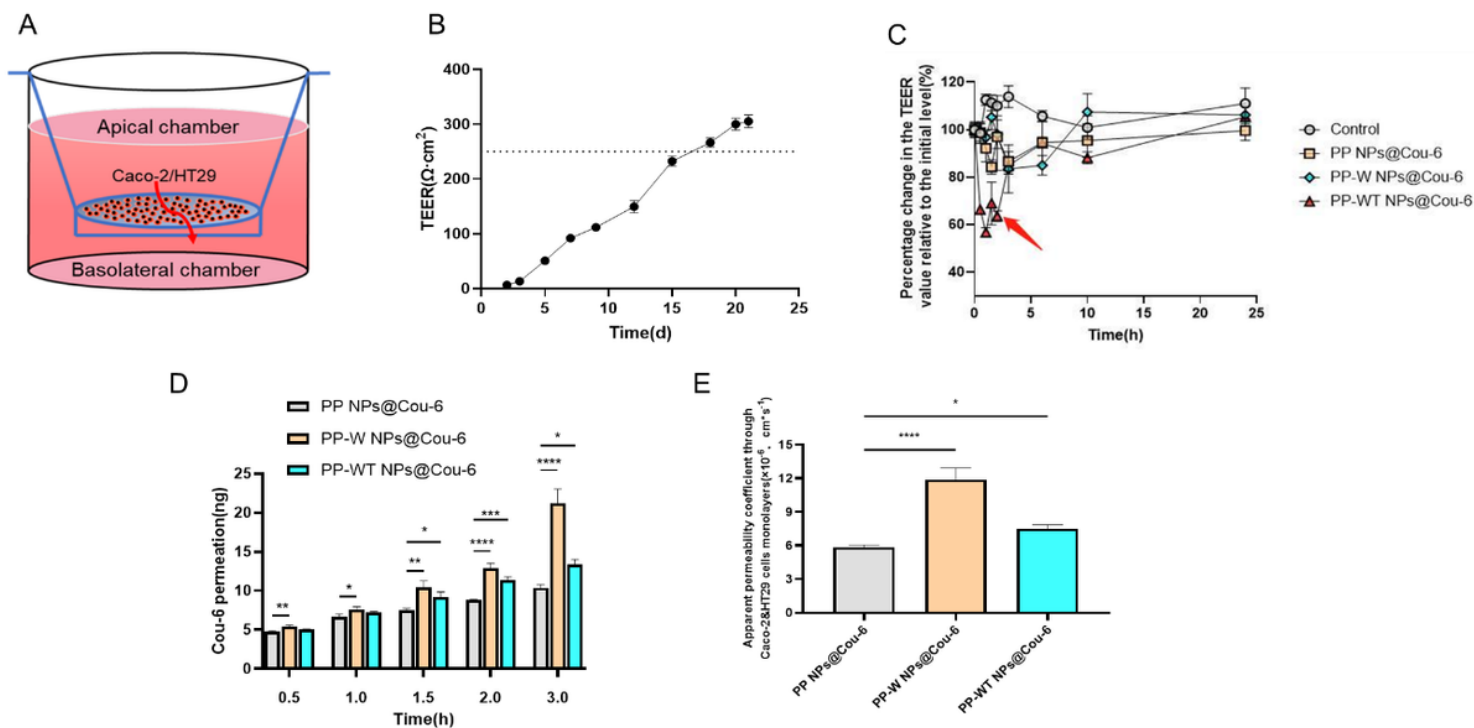


Figure 5

Transport through Caco-2&HT29 coculture cell monolayers. (A) Transwell chamber diagram. (B) TEER values of Caco-2&HT29 cells models cultured for 21 days. (C) TEER value relative to the initial level of monolayers of Caco-2&HT29 cells. (D) Cou-6 cumulative infiltration after different Cou-6 nanoparticles treatments. (E) P_{app} of different Cou-6 nanoparticles transport through Caco-2&HT29 cells monolayers. Data are presented as mean $\pm$ SD, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 (n = 3).

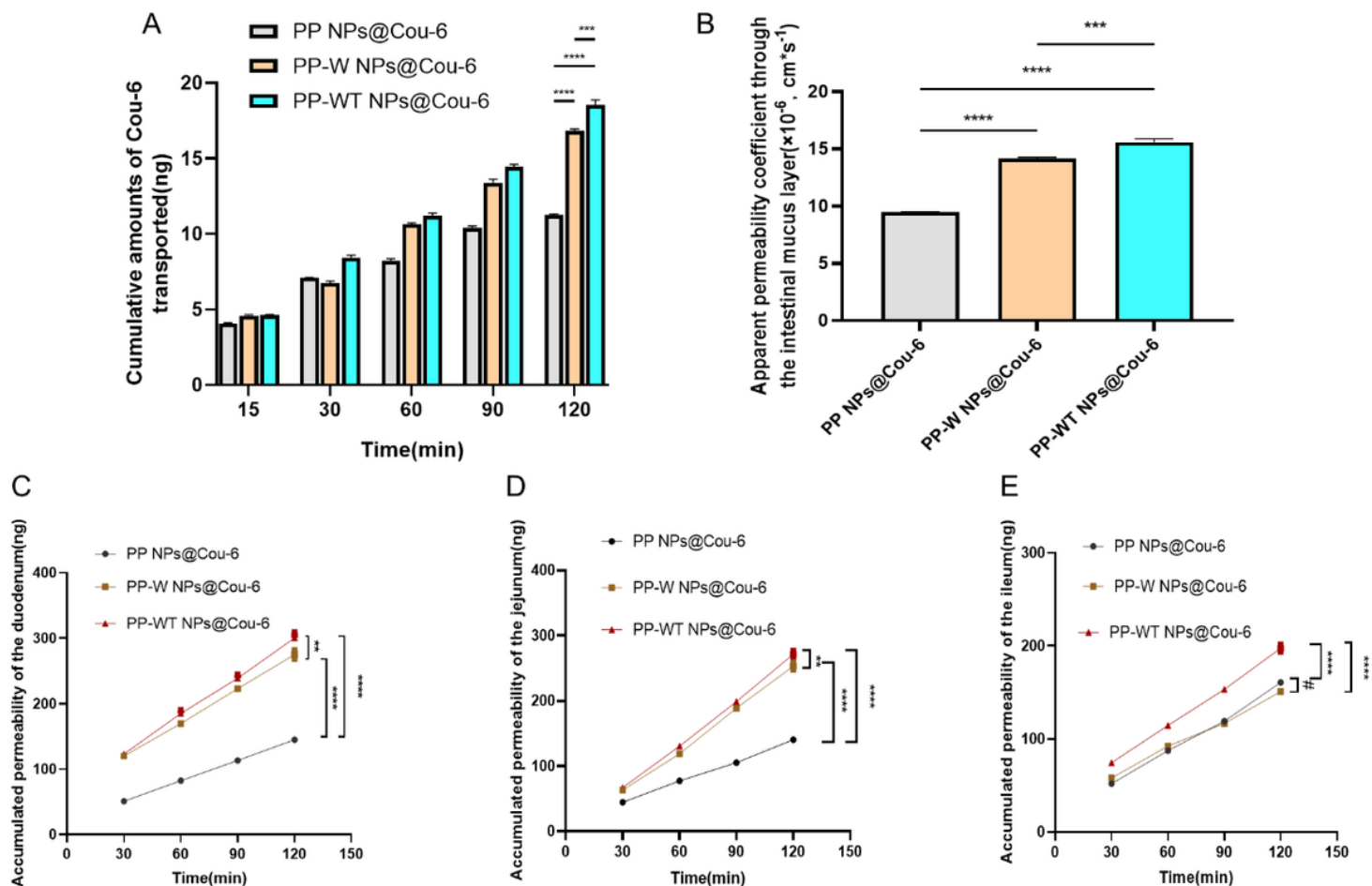


Figure 6

permeability experiment. (A) The cumulative permeability of Cou-6 through the mucus layer after different Cou-6 nanoparticle treatments. (B) P_{app} values for different Cou-6 nanoparticles transported through the mucus layer. Cumulative permeability of different Cou-6 nanoparticles through the (C) duodenum, (D) jejunum and (E) ileum. Data are presented as mean $\pm$ SD, # $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ ($n = 3$).

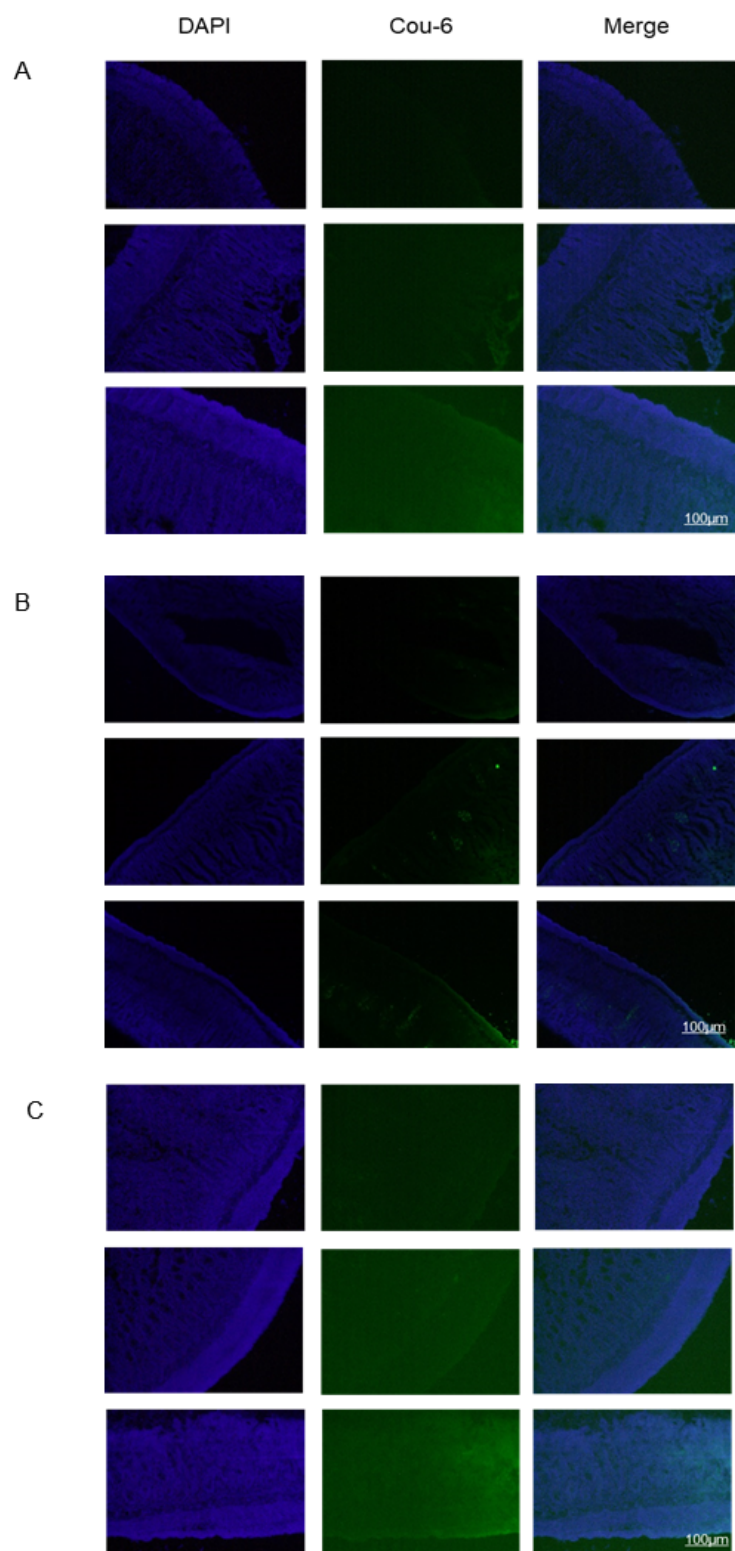


Figure 7

Observation of intestinal villi absorption at (A) duodenum, (B) jejunum, and (C) ileum. (line 1, PP NPs@Cou-6; line 2 PP-W NPs@Cou-6; line 3, PP-WT NPs@Cou-6).

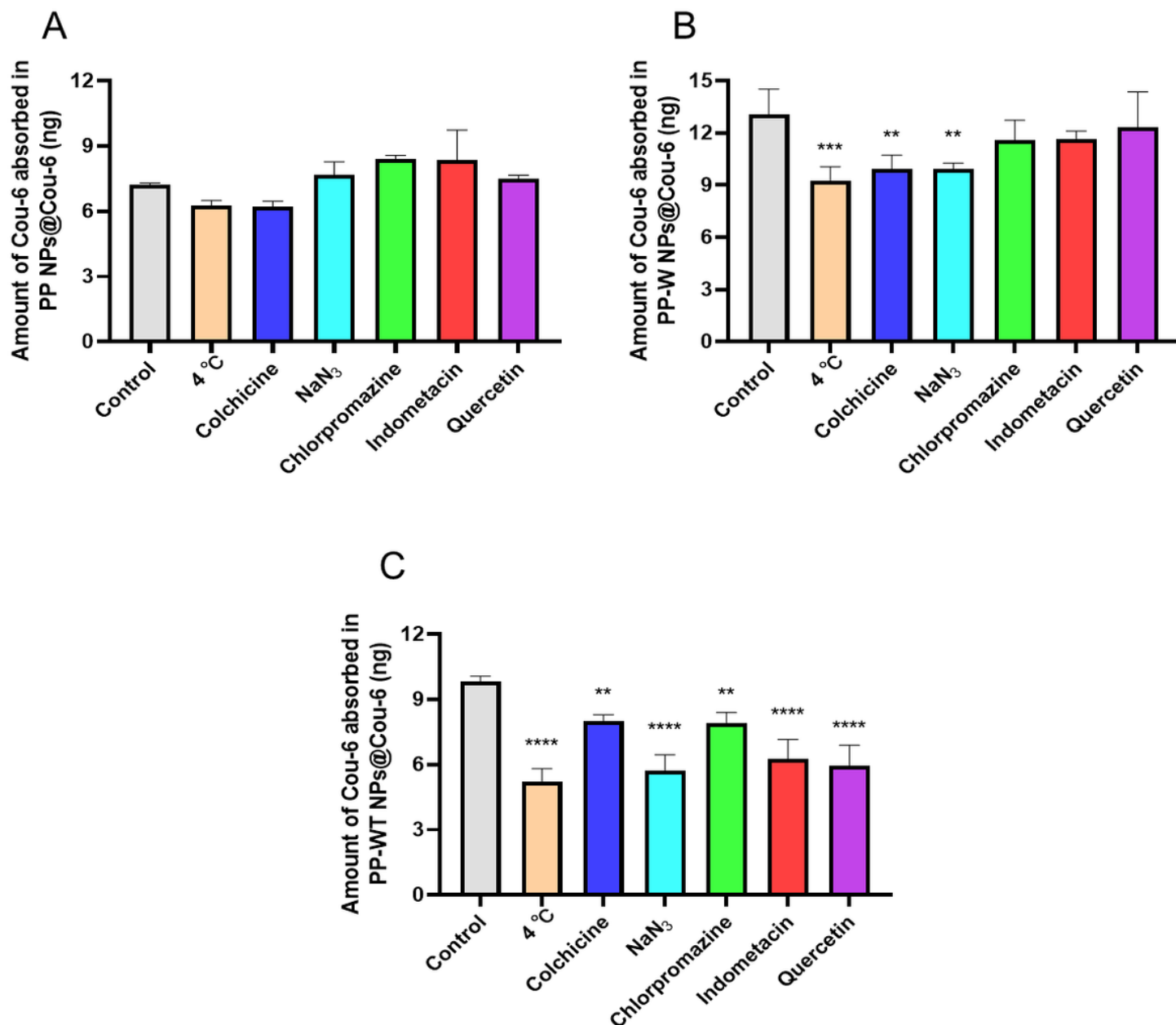


Figure 8

The intestinal absorption mechanism of PP NPs@Cou-6 (A), PP-W NPs@Cou-6 (B), PP-WT NPs@Cou-6 (C). Data are presented as mean $\pm$ SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ ($n = 4$).

Supplementary Files

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