from HT29 clone and characterized by high production of mucus, and thus an excellent model to study the nanoparticle behavior on mucosal tissue. All formulation exhibited no significant cytotoxicity on the E12 cells at the concentration from 50 to 250 μg/mL as tested using MTT assay (Supporting Information Figure S9).

For the study of cellular internalization of NPs and the influence of mucus, a procedure was performed prior to the experiment to remove the secreted mucus layer using *N*-acetylcysteine (NAC).[32] Then both pretreated and nontreated cells were incubated with different samples for 2 h, which was followed by a thorough washing process to remove the remained mucus and the attached samples.[32] The amounts of internalized F-insulin were shown in Figure 2E. All tested formulations significantly increased the cellular internalization of F-insulin compared with free F-insulin solution. NPs-1 exhibited the highest uptake among all tested samples, which was ~20-fold higher compared with free insulin, despite the status of mucus. Notably, the uptake of NPs-1 was significantly higher compared with NCs when the mucus existed, while the two samples exhibited similar amounts of uptake with the preretreatment to remove mucus. Interestingly, for NPs-1 and NPs-2, there was no significant difference between the groups with and without the preremoval of mucus, while all other formulations exhibited less amount of uptake without the pretreatment. This result suggested that mucus layer acted as an obstacle for NCs, NPs-3 and NPs-4, but not for NPs-1 and NPs-2. We then investigated the amount of NPs that were likely to be trapped in the mucus layer in the uptake study. After the incubation, the cells were treated with a mild washing process to maximally preserve the mucus or a thorough washing process to remove the mucus. As shown in Figure 2F, higher amounts of F-insulin were associated with the cells with the mild post-treatment as compared to those with post-retreatment to remove mucus for the NCs. The amount of NCs stuck in mucus was estimated to be 24.8%. However, none of the NPs exhibited significant difference between the groups of different washing procedure. For NPs-3 and NPs-4, a very interesting phenomenon is that although the mucus inhibited the cellular uptake (Figure 2F), the amount of NPs trapped in the mucus was trivial (Figure 2E). This result correlated with the mucus permeation study and might be explained by the repulsion of the mucus against the NPs.

Structural Changes of NPs. As negatively charged hydrophilic material, the pHPMA that endow the NP with "mucus-inert" property may also inhibit the cellular uptake of the NP on the epithelial cells. Therefore, the CPP-rich nanocomplex core needs to be revealed in time when the NP contacts the epithelium. The coating of pHPMA on the surface of NCs was mediated by the electrostatic interaction between the negatively charged MA-GG-OH segments on the polymer and the cationic CPP, and this noncovalent coating is dissociable over time. Therefore, we investigated the decoating process of NPs using FRET analysis. NPs prepared with fluorescent-labeled F-insulin and TRITC labeled HPMA polymers (T-pHPMA) exhibited strong FRET phenomenon in their intact form (Figure 1F). The dissociation of pHPMA from the NPs could be detected by observing the variation of the FRET intensity. As shown in Figure 3A,B, the fluorescence intensity of T-pHPMA decreased rapidly over time in mucus, while much smaller change was observed when the NPs were incubated in PBS. This phenomenon demonstrated that the pHPMA coating on the NPs was dissociable, and this process happened in a much faster rate in mucus than in PBS.

To further study the behavior and integrity of the NPs as they permeated through the mucus layer and the epithelium, the elevational distribution of different components of NPs on E12 cell monolayer was observed using confocal laser scanning microscopy (CLSM). As shown in Figure 3C, F-insulin and T-pHPMA were colocalized in the upper mucus layer at the apical side (0 μm in depth) as indicated by the overlapping of red and green signals, suggesting the NPs were in their intact form. Interestingly, less T-pHPMA was observed in deeper scanning, and there was very few colocalization from the depth of 30−60 μm. Green signals were observed in all layers from apical to basolateral side, indicating the efficient permeation of the drug through the mucus and the cell monolayer. Moreover, to study the integrity of the insulin-CPP nanocomplex core, cells were incubated with NPs prepared with F-insulin and TRITC-labeled penetratin (T-penetratin). CLSM images of E12 cells were taken and were shown in Figure 3D. A large proportion of the green and red signals were overlapped, which demonstrated that most of the insulin was still associated with the CPPs. These results implied that, as the NPs permeated through mucus, the pHPMA coating gradually dissociated from the CPP-rich nanocomplex core, while the latter remained intact and were internalized by the epithelium.

Transepithelial Transport and Intracellular Progression. Transport of F-insulin through E12 cell monolayer was evaluated using Transwell permeable supports. For each sample, the experiments were also performed with one group of cells pretreated with NAC to remove mucus. The apparent permeability coefficient (Papp) of F-insulin from apical to basolateral compartment through the cell monolayer was determined and shown in Figure 3E. All tested formulations exhibited higher Papp value compared with free insulin, which was consistent with the cellular internalized study. NPs-1 exhibited highest transepithelial transport for both pretreated and nontreated group, and was ~2.9 fold higher than that of free F-insulin. Similar to the cellular internalization study, the existence of mucus