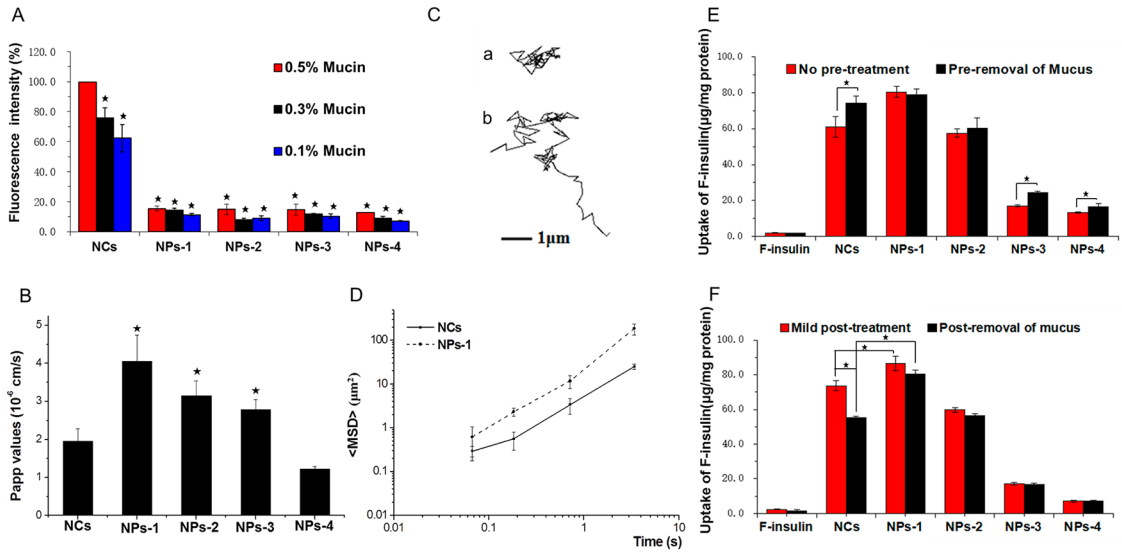

Figure 2. (A) The amount of particle−mucin aggregates formed at different concentration of mucin. The fluorescent intensity of aggregates for NCs at 0.5% mucin presented as control and normalized to 100%. Data are means ± SD (n = 3), $^{\star}p < 0.05$, *versus* to control group. (B) Papp value of the particle permeation across mucus from donor and acceptor compartments of an Ussing Chamber System. Data are means ± SD (n = 3), $^{\star}p < 0.05$, *versus* to NCs group. (C) Representative trajectories of the Brownian movement of NCs (a) and NPs-1 (b) in mucus at a time lapse of 4 s. (D) Ensemble-averaged geometric mean squared displacement ⟨MSD⟩ as a function of time scale for NCs and NPs-1 in mucus. (E) Intracellular internalization of free F-insulin or particles on E12 cell with or without a pretreatment process to remove mucus. Data are means ± SD (n = 3), $^{\star}p < 0.05$. (F) E12 cell associated F-insulin or particles after 2 h of incubation with different samples. After the incubation, the cells were treated with a mild wash to maximally preserve the mucus, or with a thorough washing process to remove the remained mucus. The amount of samples trapped in mucus was calculated as the difference between the groups of different post-treatment. Data are means ± SD (n = 3), $^{\star}p < 0.05$.

lower amounts of aggregates were formed with all tested NPs compared with the insulin−CPP NCs. For example, at mucin concentration of 0.5% (w/v), the aggregates formed in NPs-1 groups were only 15.4% relative to those of NCs. However, no difference was observed among the four tested NPs. As all NPs with HPMA coating possessed hydrophilic surface with negative charge, both of the electrostatic and hydrophobic interaction between NPs and mucin can be avoided. Therefore, higher absolute value of the negative change cannot further reduce the interaction. Then we tested the permeation of the NPs through the mucus layer using an Ussing Chamber System. Porcine intestinal mucus was amounted on a semipenetrating membrane, which separated the donor and acceptor compartments. The apparent permeability (Papp) values were calculated based on the accumulative amount of diffusion within 3 h (Figure 2B). Interestingly, although all NPs exhibited low affinity with mucin, the permeation ability decreased for NPs with higher absolute value of the surface charges. The NPs-1 exhibited the highest amount of permeation, which was 2-fold as that of NCs, whereas the permeation of NPs-4 was even lower than that of NCs. This phenomenon might be due to the electrostatic repulsion between the highly negatively charged NPs with the mucus, and correlated with those studies regarding MPP, in which electro-neutral surface was demonstrated as beneficial factor for mucus penetration.

To further investigate the behavior of the NCs and NPs in mucus, the motion of particles in mucus was analyzed using a multiple-particle tracking (MPT) method.[29] The trajectories of the particle motion were recorded (Supplementary Videos 1 and 2), and the examples of the motion trajectories in 4 s were shown in Figure 2C. The ensemble-averaged mean squared displacement ⟨MSD⟩ for NCs and NPs-1 was calculated and shown in Figure 2D. The NC exhibited a highly constrained trajectory, whereas the trajectory of NPs-1 spanned much larger distances. At a time scale of 4 s, the ⟨MSD⟩ of NPs-1 was 7.7-fold higher than that of Pen-ins NCs. The slope (α) of the logarithmic ⟨MSD⟩ *versus* time scale plots was also calculated to reflect the extent of hindrance to diffusion.[29] Unobstructed Brownian diffusion is indicated by $\alpha = 1$, whereas $\alpha < 1$ suggests increasing impediment to diffusion as α approaches 0.[30] The average α of NPs-1 was 0.70, which is significantly larger than that of NCs (0.57). This result was consistent with the Brownian trajectories shown in Figure 2C and indicated a less hindered motion of NPs.

Cellular Internalization Study. For *in vitro* evaluation of the NPs, the HT29-MTX-E12 (E12) cell line was used in order to mimic the mucosa tissue,[31] which consisted of the secreted mucus layer, as well as the absorptive epithelial cells. HT-29 is a human colorectal adenocarcinoma cell line with epithelial morphology with regard to the development of confluent monolayers and tight junction formation. E12 is a subclone isolated