polymers were recorded on VANAN INOVA 400 and Agilent 6410 Triple Quad LC/MS, respectively. The molecular weight and polydispersity index (PDI) were determined by size exclusion chromatography on a Superose 200 10/300GL analytical column (Amersham Biosciences, NJ) using a Fast Protein Liquid Chromatography (AKTA FPLC) system (Amersham Biosciences, NJ). The charge density of pHPMA was estimated *via* acid−base titration and expressed as the millimole of carboxyl associated with 1 mg of pHPMA derivatives.

Preparation and Characterization of NPs. Insulin was dissolved in hydrochloric acid (HCl, 0.01M) at a concentration of 1.0 mg/mL (0.172 mM), and then the pH was adjusted to 7.0 using sodium hydroxide (NaOH, 1 M). Penetratin was dissolved in water at a concentration of 1.0 mg/mL (0.435 mM). The penetratin solution was added dropwise to insulin solution at a ratio of 1:2 (v/v) under stirring. The mixture was stirred at room temperature for 30 min, yielding an opalescent suspension (NCs). And then, NCs suspension was added dropwise to pHPMA derivatives' aqueous solution at an equal volume. The mixture was stirred for another 30 min, obtaining another opalescent suspension (NPs). Furthermore, fluorescent-labeled nanoparticles were prepared with the same method using FITC-labeled insulin (F-insulin). F-insulin was synthesized according to the previous reported procedure.[38]

The nanoparticles were characterized for particle size and zeta potential with a Malvern Zetasize NanoZS90 (Malvern Instruments Ltd., U.K.). The morphology of NCs/NPs was examined by transmission electron microscope (TEM, H-600, Hitachi, Japan). To evaluate the encapsulated efficiency (EE %) and drug loading efficiency (DL %), nanoparticle suspensions were centrifuged at 14 000 rpm for 20 min at 4 °C. After centrifugation, the amount of insulin in supernatant was measured by a reverse-phase high-performance liquid chromatography (RP-HPLC) method (Agilent 1200 series). In adition, the amount of free F-insulin in the supernatant of fluorescence-labeled nanoparticles was measured by Varioskan Flash Multimode Reader (Thermo Fisher Scientific). The excitation and emission wavelengths were set at 488 and 516 nm, respectively. To further investigate the interaction between insulin and pHPMA derivatives, FRET analysis was performed. Tetraethyl rhodamine isothiocyanate (TRITC) and fluorescein isothiocyanate (FITC) were used as FRET pairs. TRITC labeled F-insulin loaded nanoparticles were prepared using the procedure described above. The fluorescence intensity was measured through a fluorescence spectrophotometer (Shimadzu RF-5301, Japan) with an excitation wavelength of 440 nm and the emission spectrum was recorded from 500 to 600 nm. FRET efficiency (E) and the distance between the donor and acceptor (R) were calculated as following equations:

$$E = 1 - \frac{F_{DA}}{F_D}$$

$$R = R_0 - \sqrt[6]{\frac{1}{E} - 1}$$

where F_{DA} is the intensity in the presence of the acceptor, F_D is the intensity in the absence of the acceptor, and R_0 is the FÖrster distance at 50% transfer efficiency. For FITC-TRITC, R_0 is 55 nm.[27]

Mucus Permeation of NPs. For the measurement of particle−mucin aggregates, freshly NCs/NPs were dispersed in mucin solution of different concentration of 0.1%, 0.3%, 0.5% (m/v), vortexed and incubated for 30 min at 37 °C in a shaker. The mixture was centrifuged at 1500 rpm for 2 min and the precipitates were washed with PBS twice. Then, the precipitates were treated with 200 μL of NaOH (5 M); the mixtures were incubated for 10 min, and the fluorescence intensity was measured.

The permeation of NCs and NPs across mucus was measured using an Ussing chamber.[39] Briefly, 10 μL of mucus was placed uniformly in the oblong port which was covered with membrane filters (Merck Millipore, 2.0 μm). The donors were filled with 3 mL of Krebs-Ringer buffer containing test sample (F-insulin 0.05 mg/mL). The acceptors were filled with 3 mL of blank Krebs-Ringer buffer. The solutions on both sides were continuously aerated with gas (95% O_2 and 5% CO_2), and the device was maintained at 37 °C with a circulating water bath. At the determined time points, an aliquot of sample (0.2 mL) was taken from the acceptors chamber and supplemented with equal volumes of blank Krebs-Ringer buffer. The amount of permeated F-insulin was determined *via* Varioskan Flash Multimode Reader (Thermo Fisher Scientific). The Papp was calculated using the following equation:

$$\text{Papp} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

where dQ/dt is the flux of F-insulin from donor side to acceptor side; C_0 is the initial concentration of insulin in the donor compartment, and A is the membrane area (cm^2).

The Brownian movement of particles in mucus was investigated by MPT method.[29] The NCs/NPs dispersion was added to mucus sample, then transferred to microwells, and equilibrated for 30 min at 37 °C. Particles motion in the mucus was obtained using an inverted epifluorescence microscope (Nikon Ti-E, Japan). Movies were captured at 30 frames/s for 10 s, and were analyzed with NIS Elements 4.0 software to extract the x and y positional data over time. For each trajectory, the time-averaged mean squared displacement (MSD) was calculated as a function of time scale. The ensemble-averaged MSD ($\langle$MSD$\rangle$) for all particles in the mucus sample was calculated by taking the geometric mean of the individual particles' time-averaged MSDs.

Cell Culture. HT29-MTX-E12 (E12) cell were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) with high glucose, 10% (v/v) fetal bovine serum (FBS; Sigma), 1% (v/v) nonessential amino acid, 1% (v/v) L-glutamine, and 1% penicillin and streptomycin (100 IU/mL). The cells were incubated at 37 °C in 5% CO_2. For the study of intracellular uptake, cytotoxicity, and uptake mechanism, E12 cells were seeded into 96-well plates (50 000 cells per well) and allowed to attach in growth medium at 37 °C in a 5% CO_2 incubator. For the establishment of *in vitro* cell monolayer, the E12 cells were seeded at a density of 10×10^4 cells/mL on a polycarbonate membrane (0.4 μm in pore size, diameter 6.5 mm, 0.33 cm^2 of cell growth area) in Costar Transwell 24 wells/plate (Corning Costar Corp.). Mediums in both upper and bottom compartments were changed every day. The transepithelial electrical resistance (TEER) was measured with an electrical resistance meter (Millicell ERS-2, Millipore) to monitor the integrity of cell monolayer.

Intracellular Uptake Studies. To investigate the cellular internalization of NPs and the influence of mucus, the mucus layer was removed a pretreatment process using NAC.[32] Then, the pretreated and nontreated cells were incubated with test samples (F-insulin, 0.25 mg/mL) for 2 h, which was followed by a thorough washing process to remove the remained mucus and the attached samples. Then, cell lysis buffer was added, and the cell-associated fluorescence was measured (Varioskan Flash Multimode Reader, Thermo Fisher Scientific), and total protein was determined by BCA assay kit (KeyGen Biotech Co., Ltd., Nanjing, China). The uptake amounts of F-insulin were expressed as the quantity of F-insulin associated with 1 mg of cellular protein. To further evaluate the influence of mucus on the uptake of NCs/NPs, cells were incubated with test samples (F-insulin, 0.25 mg/mL) for 2 h. Then, cells were treated with a mild wash to maximally preserve the mucus, or with a thorough washing process to remove the remained mucus.[32] Subsequently, the cell-associated fluorescence and the total protein were measured following the procedures described above.

Structural Changes of NPs. The integrity of NPs in mucus over time was investigated using FRET analysis. Briefly, the TRTIC labeled HPMA derivative and FITC labeled insulin were used to prepare NPs. The NPs suspensions (at F-insulin concentration of 0.25 mg/mL) were loaded in a 96-well plate containing mucus or PBS (pH 7.4), and gently shaken in a thermostatic rotary shaker at 37 °C. At different time intervals, the plate was irradiated at a wavelength of 474 nm and imaged at an emission wavelength of 587 nm to obtain TRITC images.

To evaluate the structural changes of NPs in E12 cells, the TRITC labeled penetratin was synthesized,[40] and used to prepare the NPs. Then, cells were incubated with the NP for