possessing cationic and hydrophobic nature in surface property.[9,10] Reduced absorption of NPs caused by the mucus barrier has recently been paid increasing attention, and different techniques have been proposed to solve this problem.[8,10−13] One excellent example is the development of Mucus Penetrating Particles (MPP) inspired by the virus that can freely diffuse through mucus.[8] It was found that NPs with a densely PEGylated surface exhibited excellent diffusion ability in mucus. The hydrophilic and electrically neutral surface of the NP serves as "mucus-inert" interface that prevents the trapping of the particles, and facilitates their diffusion through the low viscosity pores in mucus matrix.[10] However, it should be noted that mucus permeation and epithelial absorption require very different surface properties of a nanocarrier. As reported by different researchers, the hydrophilic and electro-neutral surface may prevent the interaction of NPs with the target cell membrane and, thus, may decrease their uptake by epithelial cells.[14−16] Therefore, a nanocarrier designed for oral delivery should have the ability to conquer the obstacles of both mucus layer and epithelium.

Cell-penetrating peptides (CPPs) are short peptides that can facilitate cellular internalization of various molecular cargos.[17] CPPs were also demonstrated to improve the transepithelial transport of different proteins in the forms of covalent conjugation[15] or physical mixture.[18] Previous study of our group demonstrated that nanocomplex (NC) prepared with penetratin (CRQIKIWFQNRRMKWKK), one of the most promising CPPs, effectively transported encapsulated insulin across epithelium.[19] However, the cationic property of CPPs, which plays a vital role in their cellular internalization,[20] also induced a high affinity of CPP-rich particles with the negatively charge mucin that formed the mucus matrix and, thus, reduced their absorption efficiency.

We herein developed a self-assembled NP for oral delivery of protein drug, which was designed with a novel strategy to achieve both excellent mucus permeation and transepithelial absorption. With insulin as a model drug, the NPs possessed a CPP/protein nanocomplex core and an *N*-(2-hydroxypropyl) methacrylamide (HPMA) polymer (pHPMA) derivatives coating. HPMA polymer has been explored as a hydrophilic macromolecular carrier for chemotherapeutic agents for decades,[21−24] and at least six HPMA-based therapeutics have currently progressed into phase I or phase II clinical trials.[25,26] In our study, HPMA copolymer was validated for the first time as a dissociable "mucus-inert" coating material, which assembled on the NP surface to facilitate mucus permeation, while separated in time for subsequent CPP-mediated transport through epithelium. With the screening of pHPMAs with different charge density, and the investigation of the NP behavior in mucus and on the mucus-secreting epithelial cell, we demonstrated that the NP with CPP-rich core and dissociable pHPMA coating could successfully solve the dilemma of choosing between high mucus permeation and high epithelial transport.

RESULTS

HPMA Polymer Synthesis. Different from other commonly used hydrophilic polymers (*e.g.*, PEG), the physiochemical properties of pHPMA can be easily tuned by manipulating the monomers. pHPMA derivatives were synthesized using radical solution polymerization method with negatively charged *N*-methacryloylglycylglycine (MA-GG-OH) monomer. The MA-GG-OH monomer was fed at three different ratios (5%, 10%, and 20%) in order to endow the pHPMA with different densities of negative charge, and the polymers were termed as pHPMA-1, pHPMA-2, and pHPMA-3, respectively (Figure 1A). The ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra and mass spectrograms were recorded (Supporting Information Figures S1−S5). The molecular weight of the all pHPMA derivatives was ∼45 kDa with a PDI less than 1.8 (Supporting Information Table S1). The charge density of three polymers was measured using titration method and was determined to be 0.28, 0.43, and 1.05 mmol/g, respectively (Figure 1B).

Preparation and Characterization of Nanoparticles. The nanoparticles are prepared through a two-step approach based on self-assembly strategy. Penetratin is a polycationic peptide that possesses strong affinity with negatively charged proteins.[19] Aqueous solution of insulin and penetratin were first mixed at a weight ratio of 2:1 to form polyelectrolyte nanocomplexes (NCs). Then, by adding the NCs into a pHPMA solution, the positively charged NC and negatively charged pHPMA were spontaneously assembled to form NPs with nanocomplex core surrounded by pHPMA coating, as shown schematically in Figure 1C. NPs prepared with three pHPMAs were termed accordingly as NPs-1, NPs-2, and NPs-3, and another batch of NPs (NPs-4) was prepared with a higher amount of pHPMAs-3 in formulation (Table 1). The TEM images of NCs and NPs were shown in Figure 1D. The NCs exhibited a size of 148 nm as tested by dynamic light scattering, while the size of all pHPMA coated NPs was approximately 175 nm (Figure 1E). NPs in mucus were reported to transport primarily through lower viscosity pores within the elastic matrix, and the mesh spacing ranges approximately from 10 to 200 nm.[10] Therefore, the NPs that were sub-200 nm in diameter could meet the sterical requirement for rapid diffusion. In addition, all NP exhibited insulin encapsulation efficiency above 80% and drug loading efficiency above 40%. The encapsulated insulin was released in a sustained manner within 10 h in phosphate buffered saline (pH 7.4) (Supporting Information Figure S6).

As shown in Figure 1E, the CPP-rich NCs were highly positively charged with zeta potential of 20.9 mV.