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Polyethylenimine-immobilized core–shell nanoparticles: Synthesis, characterization, and biocompatibility test

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ABSTRACT

Herein, we prepared PEI-immobilized core–shell particles possessing various types of polymer cores via a visible light-induced surfactant-free emulsion polymerization (SFEP) of three vinyl monomers: styrene (St), methyl methacrylate (MMA), and 2-hydroxyethyl methacrylate (HEMA). An effect of monomers on the polymerization and characteristics of resulting products was investigated. Monomers with high polarity can provide high monomer conversion, high percentage of grafted PEI, stable particles with uniform size distribution but less amino groups per particles. All prepared nanoparticles exhibited a core–shell nanostructure, containing PEI on the shell with hydrodynamic size around 140–230 nm. For in-vitro study in Caco-2 cells, we found that the incorporation of PEI into these core–shell nanoparticles can significantly reduce its cytotoxic effect and also be able to internalized within the cells. Accordingly, these biocompatible particles would be useful for various biomedical applications, including gene transfection and intracellular drug delivery.

1. Introduction

Polyethylenimine (PEI) is one of amine-containing polymers, widely used in biomedical fields, such as controlled release drug delivery [1], gene therapy [2,3], antimicrobial agents [4], or medical imaging [5]. Amine functional groups on PEI structure provide three main characteristics, suitable to be applied in various biomedical uses. First, the reactivity of amine functionalities is useful for further modifications or covalent couplings with biomolecules [6]. Second, they possess pH-responsive ability, which is useful for controlled release drug delivery systems [7]. Third, the cationic charge of this macromolecule is capable of binding with many negatively charged biomolecules, including DNA, RNA, and proteins for tissue engineering and gene therapy [8,9]. However, its cationic nature could cause cytotoxicity to living cells [10,11], which limits its applicable dose of usage. Consequently, there have been many attempts to improve its biocompatibility.

Cytotoxicity of cationic polymers, including PEI, is mainly contributed by their charge density and chain flexibility [11,12]. Using PEI having lower molecular weight [13], linear structure [14], and lower cationic functionality [15] has been found to possibly improve their biocompatibility. However, these could decrease some desired properties for biomedical applications, such as transfection efficiency and buffering capacity. Thus, the methods that could reduce the cytotoxic effect without drastic change on the polymers' molecular weight and functionalities are, therefore, desirable. One plausible approach for such condition is the fabrication in the form of spherical particles, such as intramolecular crosslinked polymers [16], dendrimers [11,17], and immobilized nanoparticles [8,18].

One attractive method for immobilizing PEI onto polymer particles is the one-step surfactant-free emulsion polymerization (SFEP) developed by P. Li and coworkers [19]. Such method provides core–shell particles consisting of covalently grafted PEI as a shell and poly(methyl methacrylate) (PMMA) as a core. These PEI/PMMA particles have been found promising in some biomedical applications, including gene delivery system [18], drug delivery system [7], and antimicrobial agent [20]. In addition to the lower cytotoxicity of PEI as affixed on the particle's surface, the PMMA core can be used as a reservoir for embedding some

Abbreviations: Am-P, amino groups per particle; ATCC, American type culture collection; C_{free}, concentration of PEI in diluted supernatant; CLSM, confocal laser scanning microscopy; C_m, concentration of monomer used in the reaction; C_{PEI}, concentration of PEI used in the reaction; CQ, camphorquinone; DC, degree of monomer conversion; D_{cv}, volume-average diameter of the core; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; D_n, number-average diameter; D_v, volume-average diameter; ELS, electrophoretic light scattering; FITC, fluorescein isothiocyanate; FTIR, Fourier-transform infrared spectroscopy; G_{PEI}, percentage of grafted PEI; HEMA, 2-hydroxyethyl methacrylate; M_0, molecular weight of PEI repeating unit; MMA, methyl methacrylate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N_p, number of particles per volume; PBS, phosphate buffered saline; PDI, polydispersity index; PEI, polyethylenimine; pI, isoelectric pH; PTA, phosphotungstic acid; $R_{S:C}$, shell per core ratio; SFEP, surfactant-free emulsion polymerization; St, styrene; TEM, transmission electron microscopy; TBHP, tert-butylhydroperoxide; TNBS, 2,4,6-trinitrobenzene sulfonic acid; W_{SN}, total weight of collected supernatant.

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reagents such as drugs [7], or fluorescent dyes [21]. Nevertheless, since drugs or essential reagents have different chemical properties, polarity, or other physical properties, it is quite a challenge to have appropriate core compartment to suitably accommodate those reagents.

Herein, PEI-immobilized core–shell particles with three different polymer cores: polystyrene, poly(methyl methacrylate), and poly(2-hydroxyethyl methacrylate) were prepared through an alternative one-step SFEP induced by visible light irradiation. The free radicals can be initiated by 3°-amine groups from PEI in the presence of camphorquinone (CQ). By this technique, PEI can be covalently bound as a shell of the particles and a core of vinyl polymers could be designed for specific properties and applications. The effect of three monomers with different polarities on the course of photo-induced SFEP and the properties of colloidal products were investigated. Thereafter, the PEI-immobilized particles with various vinyl polymer cores were also subjected to the biocompatibility test against the Caco-2 cell line. Furthermore, their cellular internalization was also illustrated.

2. Materials and methods

2.1. Materials

Styrene (St), methyl methacrylate (MMA), 2-hydroxyethyl methacrylate (HEMA), branched polyethyleneimine (b-PEI, 50 wt.% in water, M_n 60,000 g/mol) and camphorquinone (CQ) were all obtained from Aldrich. MMA and St were purified by distillation under reduced pressure after removing inhibitor by extraction with NaOH solution, while HEMA was purified using a column packed with aluminum oxide adsorbents (pH 7 and 9.5) obtained from Fluka.

2.2. PEI-immobilized core-shell nanoparticles via photo-induced SFEP

The procedure for the surfactant-free emulsion polymerization was described as follows. First, 5 g of PEI solution (10 wt.%) was mixed with a predetermined amount of distilled water in water-jacketed flask equipped with nitrogen inlet–outlet, water-circulating thermostat, and magnetic stirrer. The mixture was stirred with a magnetic stirrer at 600 rpm, under nitrogen gas purge for 30 min. Thermostat-controlled water (25 °C) was pumped through the jacketed flask. After that, 2 mL of purified monomer (St, MMA, or HEMA) was added and then followed by 1 mL of CQ (0.02 M, predissolved in ethanol). A total volume of reaction mixture was 50 mL. All experimental steps involving CQ were accomplished without the exposure to light before polymerization. To trigger the polymerization, the mixture was then irradiated by visible light from the 300 W light source (SP. Electric halogen floodlight with a Sylvania tubular lamp; the distance from the lamp to the center of the reactor was 25 cm) for 3 h. The colloidal products prepared from the photo-induced SFEP using St, MMA, and HEMA as monomers were abbreviated as PEI/PS, PEI/PMMA, and PEI/PHEMA, respectively. The degrees of monomer conversion (DCs) were determined gravimetrically.

2.3. Characterization of PEI-immobilized nanoparticles

Unbound PEI from the synthesized nanoparticle dispersions was removed by repeated centrifugation–redispersion cycle at 25,000 rpm for at least 2 cycles (45 min each cycle). The supernatant from each centrifugation–redispersion cycle was collected for determining the unbound PEI and a percentage of grafted PEI through TNBS assay.

Chemical functional groups of dried nanoparticles after purified by centrifugation were characterized by Fourier-transform infrared (FTIR, Perkin Elmer, PE 2000) spectroscopy using KBr disks. The spectra were recorded at a resolution of 4 cm^{-1} and 32 scans. The scanning for each spectrum was attained in a range of 4000 to 370 cm^{-1} with KBr powder as a reference background.

Number- and volume-average (D_n and D_v) hydrodynamic diameters of cleaned nanoparticles were then acquired by dynamic light scattering (DLS) method using a laser particle size analyzer (MALVERN instruments) at 25 °C. The measurements were repeated three times.

The surface charge of the centrifuged colloidal dispersions was determined using a Zetasizer (Zetasizer 3000, Malvern Instruments, UK) in 1 mM NaCl solution at room temperature. The results reported were the mean of three determinations. The pH-dependent ζ-potential of the nanoparticles was investigated by determining the ζ-potentials of the colloidal dispersions at various pH values (in a range of 3–12) adjusted by HCl–NaOH.

The core–shell morphology of PEI-immobilized nanoparticles was observed by transmission electron microscopy (TEM; JEM-1400, JEOL, 100 kV). 20 μL of 500-fold diluted sample was deposited on a copper grid and stained with phosphotungstic acid (PTA, 2 wt.%). Then, the sample was dried under a dust-free ambient environment and visualized by TEM. The information from TEM images was also used to determine volume-average diameter of the core compartment (D_{cv}) and the number of particles per volume (N_p), following Eq. (1) [22].

$$Np = \frac{\text{Total volume of core polymer per volume}}{\text{Volume of core compartment per particle}} = \frac{m_p/\rho_p}{\frac{\pi}{6}(D_{cv})^3} \quad (1)$$

where m_p is the total mass of polymerized monomer and ρ_p is the density of each core polymer assumed to be the density of the corresponding bulk polymer [23,24].

2.4. Quantitative measurement of grafted PEI

The 2,4,6-trinitrobenzene sulfonic acid (TNBS, Aldrich) assay [25,26] was slightly modified to assess the amount of free PEI in the supernatant solutions collected from the above centrifugal cleaning process. Generally, this method has been used to determine a concentration of proteins or amino acids through the reaction between the TNBS reagent and free amine groups, which yields an observable chromogenic product. First, 50 μL of the 20-fold diluted supernatant (in 0.1 M bicarbonate solution pH 8.5) was mixed with 50 μL of TNBS solution (0.02%w/v in 0.1 M bicarbonate solution at pH 8.5) in a 96-well plate. The plate was instantaneously placed in an incubator shaker (150 rpm, 37 °C) for 2 h. Finally, the reaction was quenched by 20 μL of HCl solution (1 M). The absorbance of the solution in each well in the 96-well plate was then read by an automated microplate reader (Perkin Elmer, Wallac Victor 1420) at 355 nm. The concentration of free PEI in the supernatant was determined with reference to the calibration curve of PEI solution (10–120 μg/mL) (as shown as Fig. S1 in Supporting Information). The percentage of grafted PEI (G_{PEI}) was calculated using Eq. (2) by comparing between weights of grafted PEI and total PEI used. The shell per core ratio ($R_{S:C}$), indicating the weight ratio between grafted PEI and polymer core, was determined by Eq. (3). The amino groups per particle (Am-P) was calculated by Eq. (4).

$$G_{PEI} = \frac{\text{grafted PEI}}{\text{total PEI}} \times 100 = \left[\frac{0.5 - (20C_{free} \times W_{SN})}{0.5}\right] \times 100 \quad (2)$$

$$R_{S:C} = \frac{\text{wt of PEI}}{\text{wt of PMMA}} = \frac{(G_{PEI}/100) \times (C_{PEI})}{(DC/100) \times (C_m)} = \frac{G_{PEI}}{4 \times DC} \quad (3)$$

$$Am-P = \frac{\text{total amine groups}}{\text{number of particles}} = \frac{[(G_{PEI}/100) \times (C_{PEI}/100)] \times N_A}{M_0 \times N_p} \quad (4)$$

where the total PEI of all reactions is 0.5 g, C_{free} is the concentration of PEI in diluted supernatant (g/mL), C_{PEI} are the concentrations of PEI (1 wt.%) in the reaction, C_m are the concentrations of monomer (4 wt.%) in the reaction, W_{SN} is the total weight of collected supernatant, N_A is Avogadro's number, and M_0 is the molecular weight of PEI repeating unit (43 g/mol).