Influence of Surface Coating of PLGA Particles on the Internalization and Functions of Human Endothelial Cells

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ABSTRACT: This study is focused on the uptake of PLGA particles with different coatings and its influences on the functions and toxicity of human endothelial cells. The PLGA particles coated with polyethyleneimine (PEI) or bovine serum albumin (BSA) were prepared via a one-step emulsion method, which had a similar diameter of ∼420 nm in water and ∼170 nm in a dry state but oppositely charged surfaces. Both types of the particles were readily internalized into cells within a short time regardless of their surface chemistry. Uptake of the positively charged particles caused apparently a decrease in cell viability, but did not significantly influence mitochondrial membrane potential and activity of caspase-3. The cell adhesion and migration were significantly affected, especially after uptake of the PLGA-PEI particles. The secretion levels of von Willebrand factor (vWF) and 6-k-PGF1α were not significantly influenced regardless of the surface coating.

1. INTRODUCTION

Polymeric particles in the nano and submicrometer size are promising carriers for controlled drug delivery. The lactic acid (LA) and glycolic acid (GA) copolymer, poly(D,L-lactide-co-glycolide) (PLGA),¹² is one of the most frequently used polymers in the biomedical field. Macromolecular drugs such as proteins, peptides, genes, vaccines, antigens, and cell growth factors, as well as small molecular drugs such as antibiotics, chemotherapeutic agents, and so on have been successfully incorporated into the PLGA or PLGA-based nano/micro-particles.¹

However, the inert and slightly negatively charged surface of PLGA particles limits their interactions with the negatively charged cell membrane and their intracellular uptake to some extent, which is the key step for intracellular drug delivery. So far, various attempts have been made to modify the surface of PLGA particles using cationic polymers such as dimethylldidecylammonium bromide,³ chitosan,⁴ and trimethyl chitosan⁵ to enhance their accumulation in cells and targeted organs. The relatively low surface energy causes adsorption of proteins in biological environment, leading to destabilization of the particles in physiological fluids containing salts and biomolecules. To overcome these shortcomings, molecules with antifouling properties such as poly(ethylene glycol) (PEG) and bovine serum albumin (BSA) are often decorated onto the PLGA particles. The surface characteristics such as chemistry, charge, and existence of ligand molecules not only dominate the cellular uptake and localization of particles inside cells, but also largely influence the toxicity generated by internalization of the particles. For example, Kim et al. found that branched PEI-modified PLGA particles appeared to have significant cytotoxicity to HEK293 and NIH3T3 cells than bare PLGA particles.⁶ Moreover, He et al. found dose-dependent cytotoxicity of PEG-modified PLGA particles in Chinese hamster ovary cells.⁷ In our previous study, PLGA particles were prepared by employing PEI or BSA as stabilizers, which were simultaneously loaded into/onto the particles during the formulation process. These surface-immobilized macromolecules enabled further covalent grafting of PEG and folic acid (FA), or physical modification via layer by layer (LBL) assembly.⁸–¹⁰ Introduction of the FA molecules enhances uptake of the particles by HePG2 cells, while the antifouling PEG coating reduces the nonspecific interactions between particles and proteins/cells.

The endothelial cells (ECs) reside in the interior surface of blood vessels, forming an interface between circulating bloodstream and vessel walls. They will inevitably encounter the intravenously injected particles, leading to cellular ingestion, and in turn the change of their functions. ECs are involved in many aspects of vascular biology, including control of blood pressure, blood clotting, formation of new blood vessels, leukocytes adhesion/trafficking, and inflammation. They are the
building blocks of the endothelium, which acts as a selective barrier between the vessel lumen and surrounding tissues, controlling the passage of materials and transit of white blood cells into and out of the bloodstream. Excessive or prolonged increase in the permeability of the endothelial monolayer, as in case of chronic inflammation, may cause tissue edema.

Therefore, investigation of the internalization process of colloids into endothelial cells and its influences on cell functions is of critical importance. Mitragotri et al. prepared elliptical disk-shaped PLGA particles, whose surface was further modified with polylysine. They found that the elliptical disks were endocytosed by ECs at a slower rate compared to spheres of same volume. However, both particles were eventually internalized and accumulated around the nucleus. Walker et al. demonstrated that purified single- and multimwalled carbon nanotubes triggered dose-dependent impairment of cell functions and viability of endothelial cells, accompanying with actin cytoskeleton and intercellular contact disruption. Mitragotri et al. demonstrated that purified single- and multimwalled carbon nanotubes triggered dose-dependent impairment of cell functions and viability of endothelial cells, accompanying with actin cytoskeleton and intercellular contact disruption.12

However, the uptake process of the PLGA particles with different surface coatings and their influences on the ECs’ functions are not addressed previously. In this paper, the structures and protein adsorption behaviors of PEI- and BSA-stabilized PLGA particles are first characterized. This is a very important yet often neglected issue. As recognized previously by Gil et al. the size distribution and protein adsorption of the particles in relevant biological medium can be significantly changed, which bring influences on the cell—particle interactions. Attention is then paid to their cellular uptake dynamics and subcellular distribution in human endothelial cells. The change of cell functions and toxicity are also studied in terms of cell viability, mitochondrial membrane potential, and cell adhesion, migration, and expression of adhesion-related genes. These results will unveil the insight interactions between cells and colloidal particles from cellular and molecular levels and highlight the importance of surface coating on particles for intravenous applications and intracellular drug delivery.

2. EXPERIMENTAL SECTION

2.1. Reagents. PLGA end-capped with hydroxyl groups (LA/GA = 75:25; Mn = 130 kD), branched polyethyleneimine (PEI, Mn = 25 kD), bovine serum albumin (BSA), amiodarone-HCl, amantadine-HCl, sodium azide, genistein, 4',6-diamidino-2-phenylindole (DAPI), and cytochalasin D (CytD) were purchased from Sigma-Aldrich. Sodium dodecyl sulfate (SDS) was purchased from Haotian Co., Ltd., China. BCA kit was purchased from KeyGEN Co., Ltd., China. Early endosome anti-EEA-1, LysoTracker Green, and rhodamine 123 were obtained from Invitrogen Co., Ltd., China. The caspase-3 assay kit was purchased from Beyotime Institute of Biotechnology, China. Secretion of von Willebrand factor (vWF) and 6-keto-prostaglandin F1α (6-keto-PGF1α, a metabolite of prostacyclin) was detected by enzyme-linked immunosorbent assay (ELISA) kits obtained from Guangzhou Rich Co., Ltd., China. Milli-Q water was used throughout the experiments.

2.2. Cell Lines. The human endothelial cells (CRL-1730) were obtained from American Type Culture Collection (ATCC, U.S.A.) and maintained with regular growth medium consisting of high-glucose RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, and cultured at 37 °C in a 5% CO2 humidified environment. The cells were routinely passaged at 80–90% confluence. Briefly, the culture medium was removed and the cells were carefully washed three times with PBS to remove all traces of serum, which contains trypsin inhibitor. The cells were then incubated with 1 mL trypsin/ethylenediaminetetraacetic acid disodium salt (EDTA, 0.25%) in phosphate buffered saline (PBS) until the cell layer was dispersed (usually within 5 min). The trypsin was blocked by adding 6.0–8.0 mL complete growth medium and the cells were aspirated by gentle pipetting. The cells were isolated by centrifugation at 1000 rpm for 5 min and then dispersed in fresh culture medium and incubated at 37 °C.

2.3. Particle Preparation and Characterization. The PLGA particles were prepared by an O/W emulsion-solvent evaporation method. Briefly, 2% (w/v) PLGA dichloromethane solution (organic phase) was added into 4 mL 5% PEI or 3% BSA solution (water phase) and then emulsified with an ultrasonicator (MISONIX Ultrasonic liquid Processors) for 20 s. The obtained emulsion was poured into 150 mL of water and stirred for 3 h at room temperature with a magnetic stirrer until the organic solvent was completely evaporated. The PLGA particles were collected by centrifugation at 12000 g for 15 min and washed with water five times to remove free PEI or BSA in the water phase. The PLGA particles containing Nile red were similarly prepared by adding 0.2 mg mL−1. Nile red into the PLGA solution before mixing with the PEI or BSA solution. The fluorescence intensity of NR-labeled PLGA-PEI and PLGA-BSA particles was normalized with each other in further quantitative comparison of the cellular uptake study. The concentration of the particles was determined by weighing the completely dried particles from 1 mL suspension.

The morphology of the PLGA particles was analyzed by transmission electron microscopy (TEM, Philips TECNAL-10). A drop of the PLGA particles suspension was added onto a copper grid with carbon membrane and dried in air.

2.4. Elemental Analysis. The percentage of nitrogen, hydrogen, and carbon elements in the PLGA particles were analyzed by elemental analysis (Flash EA 1112, Thermo Finnigan) to calculate the relative content of PEI and BSA in the PLGA particles, respectively.

2.5. Protein Adsorption Measurements. After the PLGA-PEI and PLGA-BSA particles were incubated in RPMI 1640 (Gibco, U.S.A.) and RPMI 1640/10% (v/v) fetal bovine serum (FBS) mediums for different time.

2.6. Cellular Uptake of PLGA Particles. The cells were cultured in a 24-well plate until 80–90% confluence and were then cultured with various concentrations of NR-labeled PLGA particles for 3 h or cultured with 50 μg mL−1 of NR-containing PLGA particles (1.66 × 10−12 and 1.63 × 10−12 mol/L for the PLGA-PEI and PLGA-BSA particles, respectively). After the cells were washed three times with PBS to remove the free PLGA particles, they were detached by trypsin. The uptake of NR-containing PLGA particles was determined by flow cytometry (FACS Calibur, Becton Dickinson). To clarify the uptake mechanism, the energy dependence of cell–particle interaction was assessed by treatment under 4 °C and with sodium azide, respectively. Different pharmacological inhibitors, including 2 mM amiodarone-HCl, 1 mM amantadine-HCl, 100 mM genistein, and 10 μg mL−1 cytochalasin D were also used to treat the endothelial cells for 1 h before incubation with the PLGA particles, respectively.

2.7. Intracellular Distribution. Fluorescent staining of early endosomes, lysosomes, cell nuclei, and mitochondria were performed to display the intracellular distribution of the PLGA particles by confocal laser scanning microscopy (CLSM, LSM 510, Carl Zeiss). Briefly, after culturing with 50 μg mL−1 of NR-containing PLGA particles for the desired time, the cells were carefully washed with PBS three times and continually cultured with anti-EEA-1, LysoTracker Green, DAPI, and MitoTracker Green at 37 °C for another 30 minutes.

2.8. Cell Mortality and Cell Viability. The endothelial cells were cultured in 24-well and 96-well plates until 80–90% confluence for cell
mortality and viability studies, respectively. The medium was replaced with a fresh one containing the PLGA particles of variable concentrations. To determine the cell mortality, after cocultured with the particles for 24 h, the cells were washed three times with PBS and then continually cultured with 50 μg mL⁻¹ PI solution for 30 min in the dark. They were finally measured by flow cytometry and analyzed by CellQuest Pro software. To determine the cell viability, after the cells were coincubated with particles for 24 h, 20 μL of 5-[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg ml⁻¹) was added to each well and the cells were further cultured at 37 °C for 4 h. The dark blue formazan crystals generated by the mitochondria dehydrogenase in viable cells were dissolved in dimethyl sulphoxide, whose absorbance was measured at 570 nm by a microplate reader (Biorad Model 680).

2.9. Cell Cycle Detection. The cells were cultured in a 12-well plate until 80–90% confluence. After treated with 10, 50, and 150 μg mL⁻¹ PLGA particles for 24 h, respectively, the cells were washed with PBS and detached with trypsin, centrifuged, and finally fixed in 75% ethanol for 1 h at 4 °C. Two PBS washes were performed before the cells were resuspended into PBS containing 50 μg mL⁻¹ PI and 0.1% RNase A for 1 h. After the cells were filtered to remove cell aggregates, the content of DNA was analyzed by flow cytometry and CellQuest Pro software. A flow cytometric assay kit. Briefly, cells were incubated with different concentrations of particles (50 and 150 μg mL⁻¹) for 24 h. The cells were first lysed to collect their intracellular contents. The cell lysates were then tested for their enzyme activity by the caspase releases the chromophore pNA, which has a high absorbance at 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the absorbance values at 405 nm detected by a microplate reader (Biorad Model 680).

2.10. Measurement of Mitochondrial Membrane Potential (MMP). After the endothelial cells (cultured in a 24-well culture plate with 80–90% confluence) were incubated with the PLGA particles for 24 h, rhodamine 123 solution was added with a (MMP).

2.11. Caspase-3 Activity Detection. The activity of caspase-3 was determined using a colorimetric assay kit. Briefly, cells were trypsinized and resuspended in PBS, and then observed under a hemacytometer. The quantification of absorbance values at 405 nm was measured by ELISA assay according to the user’s manual. Spectrophotometric readings were recorded at 450 nm, and the results were obtained by comparison with a calibration curve. Each value was averaged from three parallel experiments.

2.15. Statistical Analysis. All values were expressed as mean ± standard deviation (SD). Statistically significant value was set as p < 0.05 based on Student t test and one way ANOVA.

3. RESULTS AND DISCUSSION

3.1. Characterization of PLGA Particles. The PLGA particles were prepared by an O/W emulsion-solvent evaporation method with PEI and BSA as stabilizers in the water phase, respectively. PEI with 25 kD molecular weight was chosen due to its good performance in emulsification. The average sizes of the PLGA-PEI and PLGA-BSA particles were found to be ~420 nm in water from DLS and ~170 nm in a dry state from TEM (averaged from TEM images; low magnification TEM images are shown in Figure S1). The larger size in water is attributed to the hydrophilic PEI or BSA corona on the particle surface, conveying a core–shell structure of the PLGA-PEI and PLGA-BSA particles, as confirmed by high magnification TEM (Figure 1A,B). The shadow shells (more obvious on PLGA-BSA particles), with a thickness of several tens of nm, would be reasonably attributed to PEI or BSA, which preferably dispersed in water/organic interface during particles formation because of their amphiphilicity.12 Elemental analysis found 45 mg PEI and 58 mg BSA on/in 1 g PLGA-PEI and PLGA-BSA particles, respectively. Even ultrasonication in PBS for 4 h, the PEI (44 mg/g) and BSA amounts (57 mg/g) were not changed on the corresponding particles, confirming their good stability against harsh conditions. However, a thorough washing in sodium dodecyl sulfate (SDS) solution could remove those loosely adsorbed polymers (Figure 1C,D). Consequently, the shells became thinner on the PLGA-BSA particles and almost disappeared on the PLGA-PEI particles. Nevertheless, 40 mg PEI and 37 mg BSA were still remained on/in 1 g PLGA-PEI and PLGA-BSA particles, respectively, which account for 4% of

![Figure 1. TEM images of (A, C) PLGA-PEI and (B, D) PLGA-BSA particles before (A, B) and after (C, D) SDS treatment.](image-url)
the total mass. In previous studies, Ganguly et al. and Angadi et al. suggested that the modification process may have influence on the surface morphology of particles, 22,23 which in turn affects the interactions with cells. Thus, the surface morphology of the PLGA particles was also observed by scanning electron microscopy (SEM; Figure S2). The results show that both types of PLGA particles have smooth surface, and the surface morphology is not significantly altered after the SDS treatment.

Because the thickness of the shells is much larger than the size of single molecules (the diameter of BSA molecule is around 6.8 nm),24 they cannot be composed only by the PEI or BSA molecules, but likely by the PEI or BSA aggregates interwined with the PLGA molecules. This assumption was partially proven by the fact that a 3% BSA solution alone could form 10−20 nm particles (according to DLS) after emulsification under ultrasonication. During the particle preparation, these nanoparticles could also be generated and dispersed at the PLGA/water interfaces as stabilizer.8,9,18−20 Due to technical limitations, so far the exact distribution of PEI or BSA on the PLGA particles cannot be observed directly. Nonetheless, one can safely conclude that most of the PEI (88.9%) or BSA (63.8%) molecules are stably entrapped in/on the PLGA particles, while some molecules are loosely attached and can be removed under the assistance of surfactants.

The protective coating greatly influences the surface charge and colloidal stability of particles and, in turn, governs the cell−particle interactions and toxic potential.25 Owing to the surface domination of PEI or BSA molecules, the PLGA-PEI and PLGA-BSA particles were positively (+40 mV) and negatively charged (−20 mV) in water, respectively. When these particles are used for the study of cell−particle interactions in culture medium, what the cells really “see” is more important than the bulk materials.26 Indeed, the particle surfaces are inevitably decorated with many biomolecules, especially a selected group of proteins from the culture medium. Therefore, the cells will “see” the decorated surfaces rather than the pristine ones in water.26 As shown in Figure 2A, protein adsorption on the PLGA-PEI particles occurred prominently during 1 h incubation. The adsorbed protein amount slightly increased and reached a highest value in the range of 30−40 mg per gram particles. In contrast to the PLGA-PEI particles, the protein amount was not significantly changed for the PLGA-BSA particles in the same serum containing medium (Figure 2A) during the 24 h incubation. However, protein exchange cannot be excluded due to the so-called Vroman effect,27,28 as also partly evidenced by TEM observation (Figure S3). The different protein adsorption behaviors are surely caused by the different surface properties: the positively charged PEI molecules are easily bound with the negatively charged proteins via electrostatic attraction, while the negatively charged BSA molecules will repel the proteins of the same charge sign. Besides the adsorbed proteins, the surface charge density of the particles is another important factor for the therapeutic delivery systems due to its close association with the cellular uptake.29−32 Due to the electrostatic screening and protein adsorption in the culture medium, the surface charge might be significantly changed by environmental conditions, as confirmed in Figures 2B and S4. The zeta-potential of the PLGA-PEI particles quickly decreased from +40 to −4 mV upon being incubated in RMPI 1640 due to the charge screening as well as
the adsorption of negatively charged molecules such as glutamic acid and aspartic acid and remained stable around 0 mV in the whole experimental period (24 h). In the medium containing 10% FBS, the zeta potential of the PLGA-PEI particles further decreased to $-10$ mV due to the adsorption of negatively charged proteins (Figure 2B). By contrast, the zeta potential of the PLGA-BSA particles was kept almost unchanged around $-20$ mV in both mediums due to the coverage of BSA molecules.

As a result of the electrostatic screening in the culture medium, the charge repulsion, which stabilizes the particles in suspension, will be weakened, leading to particles aggregation. Therefore, the size of the PLGA particles was further measured in RPMI 1640 and RPMI 1640/10% FBS mediums. As shown in Figures 2C and S5, the size of the PLGA particles was generally kept unchanged in the RPMI 1640 medium, regardless of their surface properties. The size of the PLGA-BSA particles remained unchanged in the serum-containing medium. However, the size of the PLGA-PEI particles quickly increased from 420 to 1200 nm upon being incubated in the RPMI 1640/10% FBS medium and then kept constant at this value until 24 h. This huge increase of particle size implies the aggregation between particles because the typical thickness of adsorbed proteins is within tens of nanometers. The negatively charged amino acids and serum proteins will decrease the surface charge density of the initially positively charged PLGA-PEI particles and may even bind between the particles.

3.2. Cellular Uptake. To make the PLGA particles detectable via fluorescence microscopy and flow cytometry, a hydrophobic dye, Nile red (NR) was preloaded during the particle fabrication. Due to its very poor solubility in culture medium, no detectable release of NR from the particles was found after incubation in RPMI 1640/10%FBS at least for 24 h at 37 °C (data not shown). Moreover, a Transwell assay found that the cells showed very weak fluorescence signals (<10$^1$) after incubated with the isolated NR-loaded particles (no direct contact with the cells) for 24 h, confirming neglectable release or accumulation of NR in lipid organelles of the cells (Figure S6). Loading of the trace amount of NR did not bring significant influences on the particle size and morphology (Figure S7, as well as surface charge (Tab. S1), colloidal stability, and protein adsorption property. Normalization of the fluorescence intensity of each type of the NR-loaded particles was performed for the quantitative measurements by FCM. In the FCM measurement, the logarithmic fluorescence intensity of untreated cells was set between 100 and 10$^1$, and those cells with the intensity larger than 10$^1$ were considered the positive ones (the cells uptaking the PLGA particles; Figure S6).$^{33}$ As shown in Figure 3A, at a fixed coculture time of 3 h, the PLGA particles that internalized into or adsorbed onto the cells increased almost linearly along with the increase of particle concentration regardless of their surface chemistry. The significant difference was found between all concentration intervals for both the PLGA particles ($p < 0.05$). Moreover, at
each fixed particle concentration, the PLGA-PEI particles were more significantly uptaken than the PLGA-BSA particles ($p < 0.05$). When the particle concentration was fixed at 50 μg mL$^{-1}$, the cellular uptake amount of the PLGA-PEI particles increased significantly along with the coculture time, especially during the first 3 h and leveled off after 12 h. For the PLGA-BSA particles, the uptake amount increased fast during the first 6 h and then leveled off after 12 h (Figure 3B). This result reveals that cellular uptake of the PLGA particles takes place very fast. Comparatively, the PLGA-PEI particles were uptaken with a significantly faster rate and larger amount at each detection time interval ($p < 0.05$).

Besides the surface chemistry, the alteration of particle size may also play a role. As reported by Lundqvist et al., the positively charged surface would like to adsorb proteins in serum-containing fluids and the particles are tended to aggregate. $^{34}$ To clarify this point, the cell-uptake experiments are also conducted in serum-free medium where the PLGA-PEI and PLGA-BSA particles have similar sizes (both around 400 nm) but different surface charge properties. As shown in Figure 3C, the average fluorescent intensity from the PLGA-PEI particles is much higher than that of the PLGA-BSA ones in all the time points ($p < 0.05$), suggesting that the cells obviously ingest more PLGA-PEI particles than the PLGA-BSA counterpart. This result substantiates the conclusion that the cell uptake of PLGA particles is mainly governed by their surface property. Even though the aggregation occurs in serum-containing medium to a different extent, it is still a consecutive result controlled by the particles with different surface coatings. Namely, the surface charge may influence on the interactions between particles and cells through the alternation of particle size. In this case, the intrinsic determination parameter is still the surface charge property. As shown in Figure 2B, unlike the BSA-passivated and negatively charged PLGA-BSA particles, the PLGA-PEI particles are basically positively charged or almost neutral although being greatly compensated by the adsorbed proteins, leading to the easier approaching and physical adsorption of the PLGA-PEI particles onto the negatively charged cell membranes. $^{26,30}$ Another possible reason is that the cells could recognize the plasma proteins adsorbed on the PLGA-PEI particles surface, resulting in differences in cellular uptake pathways and thereby the uptaken amounts.

It is known that the extracellular substances can be transported into cells through several different pathways such as transmembrane diffusion, phagocytosis, and receptor-mediated or nonspecific endocytosis. $^{35−37}$ To determine the uptake mechanisms of the PLGA particles, special inhibitors were used to treat the cells before coculture with the particles. Although some inhibitors do have some extenuating influences on cell viability, over 80% of the cell viability was still kept (Figure S8). As shown in Figure 3D, the uptake efficiency was significantly blocked under low temperature (4 °C) or by addition of 100 mM sodium azide, suggesting that uptake of the PLGA particles is an energy-dependent process, but not through nonspecific diffusion. There are several possible endocytic pathways for internalization of exogenous particles, including clathrin-mediated endocytosis, caveolae-mediated endocytosis, macropinocytosis, and clathrin-caveolae-independent endocytosis. $^{38}$ The macropinocytosis could be blocked by amiloride-HCl due to the disturbing of Na$^+$/H$^+$ channels; $^{39}$ the clathrin-mediated endocytosis can be inhibited by amantadine-HCl by preventing budding of clathrin-coated pits; $^{40}$ the caveolae-mediated uptake would be blocked by genistein through the blocking of Src-family tyrosine kinases; $^{41}$ and the cytoskeleton could be destroyed by CytD, which has a strong impact on particle transportation in cells. Figure 3D shows that cellular uptake of both types of PLGA particles was largely blocked by all reagents, especially the amiloride-HCl and amantadine-HCl ($p < 0.05$). Therefore, internalization of the PLGA particles should be mediated by several cellular uptake mechanisms: macropinocytosis took the major role for both PLGA particles, and the clathrin-mediated endocytosis also contributed a lot for the uptake of the PLGA-BSA particles.
whose internalization was obviously blocked by amantadine-HCl. The caveolae-mediated endocytosis pathway, clathrin-caveolae-independent endocytosis pathway, and cytoskeleton also played important roles in the particle transportation in cells.

### 3.3. Intracellular Distribution

In the cellular uptake process, the exogenous particles are enclosed into endosomes initially, which mature into late endosomes or multivesicular bodies and eventually fuse with lysosomes accompanying with a significant change of pH.$^{32}$ Yue et al. demonstrated that surface charge of chitosan-based particles not only influences on the cellular uptake but also on the intracellular trafficking.$^{45}$ The internalization process and the cellular distribution of the PLGA particles were microscopically monitored by staining the early endosomes with anti-EEA-1, the lysosomal compartments with LysoTracker Green and the cell nuclei with DAPI, respectively. Because the Transwell assay already demonstrated that the NR-loaded NPs did not show visible fluorescent signal under CLSM, undesired staining of the cellular organelles can be safely ruled out and the red fluorescent signals surely represent the NR-loaded NPs.

Because the endosomes would fuse with lysosomes at a later stage, we chose 3 and 6 h to observe the distribution of the particles and endosomes inside cells. Figure 4A–D shows that the internalized particles increased along with the incubation time for both types of PLGA particles, and most of which resided inside endosomes judging from the yellow color in the merge images. The existence of PLGA particles in the endosomes confirms that the macropinocytosis and the clathrin-mediated endocytosis are the major uptake pathways (Figure 3D). The lysosomes and PLGA particles were also followed after 3 and 12 h incubation. Figure 4E–H shows that only a few particles colocalized with lysosomes (yellow color) after 3 h incubation, because most of the particles resided inside the endosomes at this moment (Figure 4A,C). After a 12 h incubation, more PLGA particles were overlapped with lysosomes (yellow color in Figure 4F,H) and others were found in the cytoplasm (red color), implying that many PLGA particles had escaped from the lysosomes. The merged pictures with fluorescent and bright field images confirmed further the cell location (Figures S9,10). Moreover, although many particles were found around the nuclei, very few colocalization signals of the particles and cell nuclei could be recorded even after 24 h coinoculation, suggesting that the particles cannot penetrate into the cell nucleus during the experimental period (Figures 5 and S11). Taking all the cellular distribution results into consideration, no obvious difference was found between the PLGA-PEI and PLGA-BSA particles in terms of the particle distributions inside the endosomes, lysosomes, mitochondria, and cytoplasm.

### 3.4. Cytotoxicity

Understanding the cytotoxicity and its mechanism of particles is of paramount importance for their applications in the biological and medical fields. So far a lot of studies have been carried out to elucidate this issue in vivo and in vitro.$^{44-46}$ Many particles are very reactive in the cellular environment because of their large surface area per unit mass. It has been confirmed that the cellular uptake of particles can induce a series of responses to cells, affecting the cell behaviors such as cell proliferation, apoptosis, adhesion, migration, and differentiation.$^{47,48}$ As a type of widely used polymer particles, especially in drug delivery, the PLGA particles are generally considered to be safe and accredited as a drug carrier by the Food and Drug Administration (FDA) of the U.S.A. However, the toxicity of particles is also largely dependent on their surface properties. For example, Kim et al. found that branched PEI-modified PLGA particles appeared to be more toxic to HEK293 and NIH3T3 cells than the bare PLGA particles.$^{6}$ Furthermore, the substantial but far-reaching cytotoxicity, such as the influences on cell functions and genotoxicity, is not fully addressed due to the complexity of the biological systems and the limitations of technology. Therefore, the effects of cellular uptake of the PLGA particles on the cell viability, mitochondrial function, protein and gene syntheses, and cell adhesion and migration are further studied. Because the cell toxicity depends strongly on the particle dose, it is taken into consideration as well.$^{53}$

First, the ratios of dead cells after exposure of the PLGA particles of various concentrations were determined by PI staining. Figure 6A shows that over 90% of the cells survived even after 24 h coculture with the PLGA particles of the highest concentration (150 μg mL$^{-1}$). However, a significantly larger number of dead cells ($p < 0.05$) were found when the cells were exposed to lower concentrations (≤50 μg mL$^{-1}$) of the PLGA-PEI particles than to the PLGA-BSA particles, revealing that PEI coating is more toxic. The cell mortality ratios became insignificant between these two types of particles at higher concentrations. Second, the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyldrazonium bromide) assay was used to assess the viability of the endothelial cells after exposure to the PLGA particles for 24 h at various concentrations. Figure 6B shows that the cell viability was mainly influenced by the surface...
coating of the PLGA particles ($p < 0.05$) rather than the particle dose. For the cells incubated with the PLGA-BSA particles, the cell viability was largely maintained compared to particle-free control in all the tested particle concentrations. The cell viability was slightly decreased along with the increase in particle concentration and reached 86.5% of particle-free control when the cells were exposed to 150 μgm L$^{-1}$ PLGA-BSA particles. However, the cell viability was obviously decreased after incubation with the PLGA-PEI particles, regardless of the concentrations, and reached 76.0% of the control at a particle concentration of 150 μgm L$^{-1}$. Therefore, internalization of the PLGA-PEI particles brings more adverse effects on the endothelial cells in terms of cell viability.

Cell cycle is a series of events that can induce cell division and duplication, comprising interphase (G1, S), synthesis (S), G2 (G2), and mitosis (M) phases. Therefore, the cell cycle analysis is powerful to reflect the influence of cellular uptake on the cell proliferation. Because G2 and M phases cannot be distinguished only by DNA content, they were put together (G2/M) to represent the cell division period. The results in Table 1 show an insignificant difference for all the samples in terms of the cell phases ($p > 0.05$), revealing that exposure of both types of PLGA particles below 150 μgm L$^{-1}$ does not influence the cell cycle.

The mitochondrion is one of the important cellular organelles that regulates cell metabolism and provides energy and also involves in signaling, cell differentiation, and cell death. Mitochondrial membrane potential (MMP) is a key indicator of mitochondrial health, which reflects the pumping of hydrogen ions across the inner membrane during the process of electron transport and oxidative phosphorylation, the driving force behind ATP production. The localization of the particles inside the mitochondria (Figure S12) possibly damages the integrity of this important cellular organ, resulting in cytotoxicity to some extent. To clarify this issue, the cells were stained by rhodamine 123, and MMP assay was qualitatively performed by optical microscopy (Figure S13), quantitatively by FCM, and normalized to that of control (Figure 6C). Surprisingly, the MMPs of the cells cocultured with the particles were slightly decreased but still comparable with that of the particle-free control without significant difference ($p > 0.05$) at all the determined time intervals, indicating that uptake of both types of PLGA particles does not significantly induce the loss of MMP and integrity of mitochondrion.

Figure 6. (A) Percentage of dead cells and (B) relative cell viability normalized to that of control as a function of particle concentration with a culture time of 24 h, respectively. (C) Normalized fluorescence intensity related to the mitochondrial membrane potential of endothelial cells was quantified by flowcytometry. A total of 5 μgm mL$^{-1}$ of rhodamine 123 was used to stain cells at determined time intervals. (D) The relative activity of caspase-3 as a function of particle concentration with a culture time of 24 h. The particle-free cells were used as control; *indicates a significant difference at the $p < 0.05$ level.

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<th>G2/M (%)</th>
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</tr>
<tr>
<td>PLGA-PEI (50 μgm L$^{-1}$)</td>
<td>46.2 ± 1.3</td>
<td>34.5 ± 2.5</td>
<td>19.3 ± 1.5</td>
</tr>
<tr>
<td>PLGA-PEI (150 μgm L$^{-1}$)</td>
<td>48.1 ± 3.6</td>
<td>32.8 ± 3.5</td>
<td>19.0 ± 0.1</td>
</tr>
<tr>
<td>PLGA-BSA (10 μgm L$^{-1}$)</td>
<td>42.2 ± 1.8</td>
<td>39.3 ± 0.8</td>
<td>18.4 ± 1.1</td>
</tr>
<tr>
<td>PLGA-BSA (50 μgm L$^{-1}$)</td>
<td>44.7 ± 2.5</td>
<td>37.9 ± 2.0</td>
<td>17.4 ± 0.5</td>
</tr>
<tr>
<td>PLGA-BSA (150 μgm L$^{-1}$)</td>
<td>46.3 ± 1.7</td>
<td>36.6 ± 1.3</td>
<td>17.1 ± 0.5</td>
</tr>
</tbody>
</table>
ways, was studied after the treatment with PLGA particles. The results (Figure 6D) show that the intracellular activity of caspase-3 was not significantly altered after the cells were incubated with a high concentration of both types of PLGA particles for 24 h. The preservation of caspase-3 activity and the integrity of MMP confirm again that the PLGA particles do not elicit cell apoptosis.

3.5. Cell Adhesion and Wound Healing. Cell adhesion is very important in governing a variety of cellular functions including cell growth, migration, differentiation, survival, and tissue organization. To study the influence of PLGA particle uptake on cell adhesion, the cells were exposed to 50 μg mL\(^{-1}\) PLGA particles for 24 h, detached, and then allowed to adhere for another 3 or 24 h, respectively. Figure 7A shows that the attachment behaviors of the particle-exposed cells were significantly weakened, especially for the PLGA-PEI particle-exposed ones. Compared with that of the control, the relative percentages of the adhered cells after a 3 h incubation were significantly decreased to 50 and 72% \((p < 0.05)\) after the cells were pretreated with PLGA-PEI and PLGA-BSA particles, respectively. The relative percentage of the adhered cells after a 24 h incubation was increased to 82% for the PLGA-PEI particles pretreated cells, but was still significantly lower than those of the control and the PLGA-BSA particles pretreated cells \((p < 0.05)\). It is obvious that the particles with PEI coating cause more impendence on adhesion of the endothelial cells. Considering that the PLGA-PEI particles have a very small influence on cell viability and MMP (Figure 6), some other mechanisms, for example, regulation of the related gene expression level (as shown in Figure S14) or dysfunction of the cytoskeleton, might be involved in the impairment of cell adhesion.

The wound healing and tissue regeneration is an important physiological process, including many biological events such as cell proliferation and cell migration. In this study, the “scratch” wound closure assay was performed to examine the wound healing ability. The endothelial cells were cultured until 80% confluence and then continually cultured for 18 h with or without the PLGA particles, respectively. After the cell monolayer was scratched to form a linear wound, it was continually cultured for another 48 h in particle-free medium. Figure 7B shows that the particles-exposed cells had a weaker ability to heal the wound, especially for the PLGA-PEI exposed ones. For instance, 48 and 52% of the wounds were healed by the PLGA-PEI and PLGA-BSA particle-exposed cells, respectively, compared to the 58% wound healing of the control at 24 h \((p < 0.05)\). After 48 h, the wound healing (85%) of the PLGA-BSA particle-exposed cells was close to that of the control (87%; \(p > 0.05\)) and was significantly larger than that of the PLGA-PEI particles-exposed ones (74%; \(p < 0.05\)). The slower wound healing of the PLGA-PEI particle-exposed cells should be caused by both the slower cell migration and the poorer cytoviability (Figure 6), because the wound healing process involves both cell proliferation and migration.

3.6. vWF and 6-k-PGF\(_{1\alpha}\) Syntheses. Von Willebrand factor (vWF) is an adhesive glycoprotein synthesized exclusively in endothelial cells and megakaryocytes. Endothelial vWF affects the platelet adhesion and aggregation, blood coagulation, and fibrinolysis. Prostacyclin (PGI\(_2\)), a prostanoid, is an inhibitor of platelet activation and a powerful
vasodilator, affecting platelet aggregation and disaggregation, blood coagulation, and circulation. 6-k-PGF\(_{1\alpha}\) is a stable hydrolysis product of PGI\(_2\). Therefore, the functions of endothelial cells can be assessed through the levels of vWF and 6-k-PGF\(_{1\alpha}\) as well as cell adhesion and mobility. However, many other cell functions are influenced by the internalization and toxicity of PLGA particles, suggesting exposure to the PLGA particles may influence a part of functions of the endothelial cells. Further studies are required to find out which functions of the cells are influenced as a result of the ingestion of particles and its molecular mechanism.

3. CONCLUSION

The PLGA particles with PEI and BSA coatings were used to study their interactions with human endothelial cells. The PEI and BSA molecules were stably bound on the particles, contributing to about 4% of the particle mass. The PLGA-BSA particles could disperse well in cell culture medium containing 10% FBS and repel further protein adsorption, while the PLGA-PEI particles quickly aggregated to some extent in the cell culture medium and adsorbed a lot of proteins. Both types of particles were internalized into the cells within a short period of 24 h culture in vitro. The cell viability, adhesion, and wound healing abilities of the cells decreased to some degree after exposure to the particles, the endothelial cells maintained their cell cycle, mitochondrial membrane potential, and their functions of specific protein synthesis after exposed to the PLGA particles, suggesting exposure to the PLGA particles may only influence on a part of functions of the endothelial cells. The size, surface zeta potential, and morphology of NR-loaded PLGA-PEI particles were not significantly different from those of pure PLGA-PEI particles (p > 0.05), except that 50 \(\mu\)g mL\(^{-1}\) PLGA-PEI particles induced higher vWF secretion (p < 0.05) and 10 \(\mu\)g mL\(^{-1}\) PLGA-PEI particles induced higher 6-k-PGF\(_{1\alpha}\) secretion (p < 0.05). Although the viability, cell adhesion, and wound healing abilities of the cells decreased to some degree after exposure to the particles, the endothelial cells maintained their cell cycle, mitochondrial membrane potential, and their functions of specific protein synthesis after exposed to the PLGA particles, suggesting exposure to the PLGA particles may only influence on a part of functions of the endothelial cells. All the results indicate that surface properties of PLGA particles not only strongly influence the internalization and cell viability, but also some essential cellular physiological functions such as cell adhesion and mobility. However, many other cell functions are not obviously influenced by the internalization of particles, suggesting the necessity of developing a systematic evaluation platform to study the profound and substantial influences on cell functions. Our findings also imply that comprehensive assessment of the internalization and toxicity of colloidal materials is necessarily carried out before their applications in biological fields, in particular, for in vivo applications.

**ASSOCIATED CONTENT**

**Supporting Information**

The size, surface zeta potential, and morphology of NR-loaded PLGA particles; transwell assay; cytotoxicity of inhibitors; distribution of PLGA NPs in different cell organelles; and gene expression of cell adhesion-related proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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